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13. ABSTRACT ***(Maximum 200 Words)***

We report the cloning and characterization of a novel cDNA, OKL38. OKL38 gene expression was induced by pregnancy and hormone associated with pregnancy such as hCG. OKL38 expression was low in breast cancer cell lines and barely detectable in DMBA-induced breast tumours. Transfection of human MCF-7 breast cancer cells with OKL38 cDNA lead to reduction in proliferation and tumour formation in nude mice. The data suggest that the *OKL38* may play a crucial role in the growth regulation and differentiation of the breast epithelial cells during pregnancy and tumorigenesis. We also report that coadministration of ICI 182780 and testosterone enanthate caused mammary gland diffrentiation and reduced tumour incidence, number of tumour bearing and tumour size. The data may be useful in designing novel strategies for breast cancer prevention and/or treatment based on maximizing mammary epithelial cell differentiation

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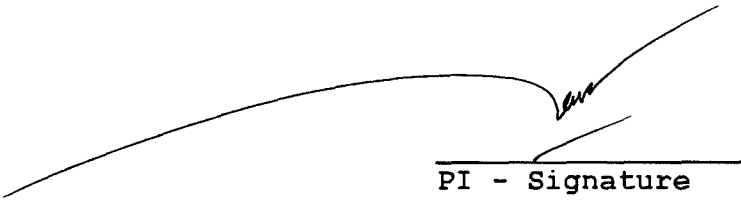

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Characteristics of Uterine Derived Growth Inhibitor (UDGI):
A Novel Growth Inhibitor of Estrogen Receptor Negative Breast Cancer Cells

Table of Contents

INTRODUCTION

Breast cancer is the most common cancer and the second cause of cancer death in women. Approximately one in nine North American women living to 80 years will develop breast cancer (1). Globally, the incidence of breast cancer appears to be increasing and an annual worldwide incidence of over one million is predicted by the turn of this century (2).

Epidemiological studies have demonstrated that for women early age at menarche, late age at first pregnancy and late age at menopause tend to increase the risk for breast cancer (3,4). The lifetime risk of breast cancer is 2 to 5 times higher in women who have a first pregnancy after age 30 than in women whose first pregnancy is at an age younger than 20 (3,4). It has been hypothesized that first pregnancy at a young age may differentiate breast cells early in life, after which they would become less susceptible to carcinogens (5-7). This hypothesis was supported by the observation that in animal models, mammary tumorigenesis is facilitated when the administration of carcinogen precedes pregnancy, however it decreases when the carcinogen exposure occurs during pregnancy (8). Normal and prolonged lactation in mice and in rats is also recognized to result in a decrease in the incidence of spontaneous or carcinogen-induced mammary tumours and an increase in tumour age when compared with forced breeding without lactation (8). In accordance, mammary DNA synthesis is at a very low level during lactation in mice and rats. These observations in experimental animals show protection of pregnancy and lactation against mammary tumorigenesis.

Both animals and humans are constantly exposed to carcinogenic agents during their life time. It is conceivable, therefore, that the longer the total period of low mammary DNA synthesis, i.e., proliferative mitotic rest, owing to pregnancy, lactation, etc., the smaller is the risk of mammary malignancy. In human, only during the first trimester of pregnancy, breast parenchymal growth and DNA synthesis are pronounced. The latter half of pregnancy is the period of proliferative and mitotic rest and breast parenchyma shows only minor DNA synthesis (9). The protective effects of having children early in life may accrue by causing breast cells to become more differentiated. Differentiation restricts the ability of a cell to grow abnormally, change its type and survive in other types of tissues. It is impractical to suggest early pregnancy as a breast cancer prevention strategy, but an investigation of the physiological basis of this protection may lead to novel risk reduction strategies.

In women, a high proportion of primary breast cancers possess the estrogen receptor (ER) and require estrogen or estrogenic activities for tumour growth. Current therapies have been directed toward interruption of estrogen by oophorectomy or the use of antiestrogens (10-12). The antiestrogen drug tamoxifen improves the survival of women with breast cancer, and has proven to be clinically useful for the treatment of metastatic ER-positive tumours (10,11,13). The pure antiestrogen ICI 182780 (ICI) (12) exhibits effects on some patients following disease progression on tamoxifen (10). ICI has recently been shown to act as a growth inhibitor even in the complete absence of estrogen stimuli (14,15), and to actively regulate gene expression in a direction opposite to that of estrogens (15) and lacks tamoxifen's uterine side effects (16).

Androgen receptors are found in 85% of all breast cancer specimen investigated (17). Androgens have been used for the treatment of breast cancer in women (18). Antiproliferative effects of

androgens on the growth of certain breast cancer cell lines have been reported (17). In addition, DHEA exerts a potent inhibitory effect on the development of DMBA-induced mammary carcinoma in the rat (19,20). It has been shown that combination of estrogen and testosterone can induce a high incidence of breast cancer (21).

Peptide growth factors and inhibitors play key roles in regulating the proliferation of normal breast epithelium (22). The importance of peptide growth factors in the pathogenesis and behaviour of breast neoplasms is evident in the large amount of literature that has accumulated in the past decade concerning the roles of EGF, IGFs, TGF-α, TGF-β, and FGF (22, 23). To date, the best characterized inhibitor is TGF-β (24). A negative regulatory function for insulin-like binding protein 3 (15, 25-27), and for mammary derived growth inhibitor (28) have been reported. Abnormal expression of growth factors and growth inhibitors has been implicated in tumorigenesis (28-30). These observations suggest that interruption of growth factor action (or production) or enhancement of growth inhibitor production by breast cancer cells would represent new strategies for arrest of tumour growth.

EXPERIMENTAL PROCEDURES

mRNA differential display: Differential display was performed using RNA from pregnant mammary gland according to the protocol supplied with the RNAmap^{TM} kit (GeneHunter Corp., Nashville, TN). Briefly, 5 μg of DNAse I-treated total RNA were reverse transcribed with $T_{12}M^n$ (where n may be G, A, T, or C), followed by PCR amplification in the presence of [α-^{33}P]dATP (NEN) using the corresponding $T_{12}M_n$ primer, downstream, and one arbitrary primers supplied with the kit, AP_1-AP_5, upstream. The PCR-amplified fragments were separated on 6% denaturing polyacrylamide gel. The gel was dried and exposed to Kodak XAR film, and cDNAs representing differential expressed mRNAs were excised from the dried gels and reamplified cDNA fragments were used to as probes in Northern blotting to verify their differential expression in mammary gland. The differential probe was used to screen human ovarian cDNA library as described (31). The isolated human cDNAs were sequenced by the Sanger dideoxy chain determination method and their nucleotide sequences were compared with those deposited in the Genbank and EMBL data banks.

Animals and drug administration. Animal experiments were approved by local Animal Care Committee. Female Sprague-Dawley rats, 50 days old at the beginning of the experiments, were obtained from Charles River, Quebec. To study changes in OLK38 expression during pregnancy and lactation of normal rats, pregnant rats were sacrificed on days 0, 4, 10, 16, and 21 of pregnancy and day 3 of lactation. Mating dates were established from the appearance of vaginal plugs. Day 1 of pregnancy was the day on which a plug was observed. The animals were sacrificed and the mammary gland collected as described above.

To study the effects of human chorionic gonadotropin on OKL38 expression, rats were inoculated by i.p. injection with doses of 10, 20 and 40 UI of hCG/day in 200 μl of PBS for 21 days in a manner such as to simulate levels seen at time of lactation as described (32). Control rats were administered 200 μl PBS. After the last injection, the rats were allowed to rest for an additional 7 days and at the end of the experiment they were sacrificed. Breast tissue was assayed for OKL38 mRNA Northern blotting.

To study the effects of antiestrogen ICI 182780 and testosterone enanthate on growth and differenciation. Fifty day old female Sprague-Dawley rats (Charles River, Montreal, Quebec) were divided into 4 groups (12 animals per group). On the first day of the experiment, the animals of the appropriate groups were underwent isoflurane-induced anaesthesia. Animals were implanted with 0.5 cm silastic tube (0.125 in (outer diameter), and 0.062 in (inner diameter), Dow Corning, Midland, MI) either empty or containing testosterone enanthate (TE) (Sigma) inserted sc in the dorsal area of each animal of indicated groups. The animal were treated for 3 weeks with the following: 1) empty silastic tube and castor oil; 2) testosterone enanthate and castor oil; 3) empty silastic tube and preformulated ICI 182780 in castor oil (Zeneca Pharmaceuticals, England) at a dose of 1 mg/kg BW, once per week; and 4) ICI (1 mg/kg/week) plus TE (1.2 μg/day). The dose of ICI 182780 was based on our previous studies. The rate of TE released per day was 1.2 μg. The proliferative marker Ki-67 and differentiative markers whey acidic protein (WAP), mammary derived growth inhibitor (MDGI), α- and ß-caseins were used as surrogate measure of treatment success.

Induction of mammary tumours by DMBA. We used the standard DMBA-induced mammary tumour experimental model (33) to study the expression of OKL38 gene during pregnancy. Mammary carcinomas were induced by a single intragastric administration of 20 mg dimethylbenz(A)anthracene (DMBA, Sigma Chemical Co., St. Louis, MO) in 1 ml peanut oil at 50-52 days of age. This standard procedure yields palpable (> 0.5 cm) tumours in about 75% of animals by day 80 following carcinogen administration.

To determine OKL38 expression in DMBA-induced mammary tumour during pregnancy. Rats bearing DMBA-induced breast tumours were mated. Mating dates were established from the appearance of vaginal plugs. Pregnant rats were sacrificed on day 16 of pregnancy and tumours were collected.

To study the effects of TE and ICI on DMBA-induced tumour incidence, tumour size and tumour number. Mammary carcinomas were induced by a single intragastric administration of 20 mg DMBA (Sigma Chemical Co., St. Louis, MO) in 1 ml peanut oil at 50-52 days of age. Rats were divided into 4 groups (n=20) and treated as follows 1) empty silastic tube and castor oil; 2) TE and castor oil; 3) empty silastic tube and ICI 182780 in castor oil (Zeneca Pharmaceuticals, England) at a dose of 1 mg/kg BW, once per week; and 4) ICI (1 mg/kg/week) plus TE (1.2 μg/day) one day before the oral administration of DMBA. Tumour size and number were recorded. The two largest perpendicular diameters of each tumour were recorded with callipers to estimate tumour size. Rats were killed 150 days after DMBA administration. The uteri, ovaries and mammary tissue were removed for later analysis. Analysis of the incidence of development of mammary tumours was performed using the Fishers exact test (34). The data are presented as the mean ± SEM.

Breast cancer cell lines and transfection study.

To determine the OKL38 gene expression in human breast cancer cell lines, MCF-7 , T47D,

ZR75, MDA-231, Hs578T, and HBL-100 were grown to 90% confluence. Poly "A" RNA was purified and Northern blotting was performed to determine the levels of OKL38 mRNA.

To generate MCF-7 cell stable transfectant cell lines, the entire coding region of OKL-38 cDNA was cloned into mammalian expression vector pcDNA3.1. MCF-7 cells were seeded at 2x 10^5 in 100 mm culture dishes in 90% α-MEM (Life Technologies, Inc.) containing 10% FCS with Garamycine 24 h prior to transfection. Cells were transfected with 5 μg of full-length OKL38 cDNA (pcDNA3.1-OKL38) or pDNA3.1 control plasmid DNA and 28 μl of Lipofectamine reagent (Life Technologies) following recommendations of the manufacturer. 48h following transfection, cells were split 1:10 and replaced with growth medium containing 800 μg/ml G418 (Calbiochem, La Jolla, CA). After 4 weeks, clones were isolated, expanded and assayed for OKL38 expression by Western and Northern blot analyses.

Cell number refers to mean cell number counted by hemocytometer 8 days after seeding 2.5x10^4 cells in wells containing α-MEM supplemented with 10% fetal calf serum. Means were determined from quadruplicate replicate wells and in no case did standard deviation exceed 15% of the mean value.

In vivo tumor formation was assayed using 4-8 week old athymic nude mice (CD1 nu/nu, Charles River). Each cell line was assayed in four mice, and each mouse received an injection of 5x10^6 cells into an inframammary fat pad, and another identical injection of the same cell line into a contralateral fat pad. 30 days after injection, animals were inspected for grossly visible tumours.

Statistical analysis: Differences in OKL38 gene expression were analysed by Student's t-test. Differences in cell number and tumor number between parental lines and transfectants were tested using the Mann Whitney U-test.

Generation of an OKL38 antibody- Synthetic peptides corresponding to predicted amino acids 243 to 267 of the human OKL38 (N- **arg glu gln ser ile leu ser pro ser pro tyr glu gly tyr arg ser leu pro arg his gln leu leu cys phe** -C) were synthesised and coupled to preactivated keyhole limpet hemocyanin (Sheldon Biotechnology, Montréal, Quebéc). Rabbit polyclonal antibodies were produced according to standard protocols. Affinity purified serum following the sixth boost was used in these studies.

Western Analysis: Mammary tissue were homogenized in buffer containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1% NP-40, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μM PMSF, and 100 μM $NaVO_4$. Cells were lysed in the above buffer. Cell lysate was used to determine changes in the levels of OKL38 by Western blottings were as described (35). Blots were incubated with rabbit anti-OLK38 (1:500 dilution) and horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody (1:7500). Blots were visualized with a chemiluminescent detection system (ECL, Amersham) and exposed to film for 10 sec to 45 sec.

Northern Blot: To investigate the effects of testosterone enanthate and ICI 182780 on the gene expression of milk protein genes and MDGI, total RNA was isolated from tissues as described (36). Northern blots were performed on total RNA and blots were hybridized with mouse α-

casein, mouse ß-casein, mouse WAP and mouse MDGI (37). To detect OKL38 mRNA, poly "A" RNA was isolated from indicated tissues of female rats as described (36). Northern blots were performed on poly "A" RNA or total RNA and blots were hybridized with human OKL38 cDNA as previously described (36). To control for equal loading of wells, blots will be rehybridized with GAPDH cDNA (ATCC). Quantitative analysis of gene expression is accomplished by scanning autoradiograms densitometrically. The figure was adjusted for minor differences in the amount of RNA loaded.

Immunohistochemistry and histology: mammary tissue was immersed in a solution of 10% buffered formalin. After fixation, the mammary tissue was routinely processed in a tissue processor and embedded in paraffin. Sections of 5 μm were cut and stained with haematoxylin-eosin. Examination of the slides were performed by light microscopy. The ImmunoCruz Staining System was used for immunohistochemical study. Briefly, the slides were deparaffinized, rehydrated in water and incubated with 3% H_2O_2 for 20 min to block endogenous peroxidase activity. To examine expression of Ki-67, antigens were retrieved by heating the slides in citrate buffer (pH 6) for 5 min. After preincubation with normal serum for 20 min at room temperature, the primary antibody was applied (2 μg/ml) and incubated overnight at 4 ^{0}C. The section then incubated with the appropriate biotinylated secondary antibody at 1:500 dilution followed by peroxidase-conjugated streptavidin complex according to the manufacturer's instruction and DAB. The section then counterstained with haematoxylin. To evaluate the Ki-67 labelling index, in each group of 500 epithelial cells were counted in randomly chosen fields at x 400 magnification. The Ki-67 labelling index was expressed as the number of clearly labelled Ki-67 reactive nuclei in 500 cells counted. Significance difference was determined by Student's t-test.

RESULTS

Part 1: Cloning and characterization of a novel pregnancy induced growth inhibitor in mammary gland.

RNA derived from mammary tissue of non-pregnant and pregnant rats was subjected to differential display. Among 18 diffrential bands obtained, one band of approximately 450 bp was novel as determined by sequence analysis. The differential expression of this particular cDNA was confirmed by Northern blotting. The 450 bp probe detected an approximate 1.6 kb mRNA species in the human, rat and mouse mammary tissues (data not shown).

In order to clone the full length human cDNA, human ovarian cDNA library was screened using this 450 bp probe. Eight positive clones were isolated and clone-purified. One clone containing an approximate 1.6 kb insert was isolated and sequenced by the Sanger dideoxy chain termination method.

Comparison of the nucleotide sequence against the non-redundant nucleotide database of Genebank established this 1.6 kb cDNA was novel. Blast search revealed no significant homology with any known sequences. This cDNA (Genebank accession no AF191740) contained 1607 bp and was full length cDNA. An initiator ATG codon (position 127) is followed by a single open reading frame of 317 amino acids with a calculated molecular weight of 34.5

kDa (Fig. 1). The open reading frame ended with a TGA terminator codon at position 1078 followed by 529 nucleotides in the 3' untranslated region.

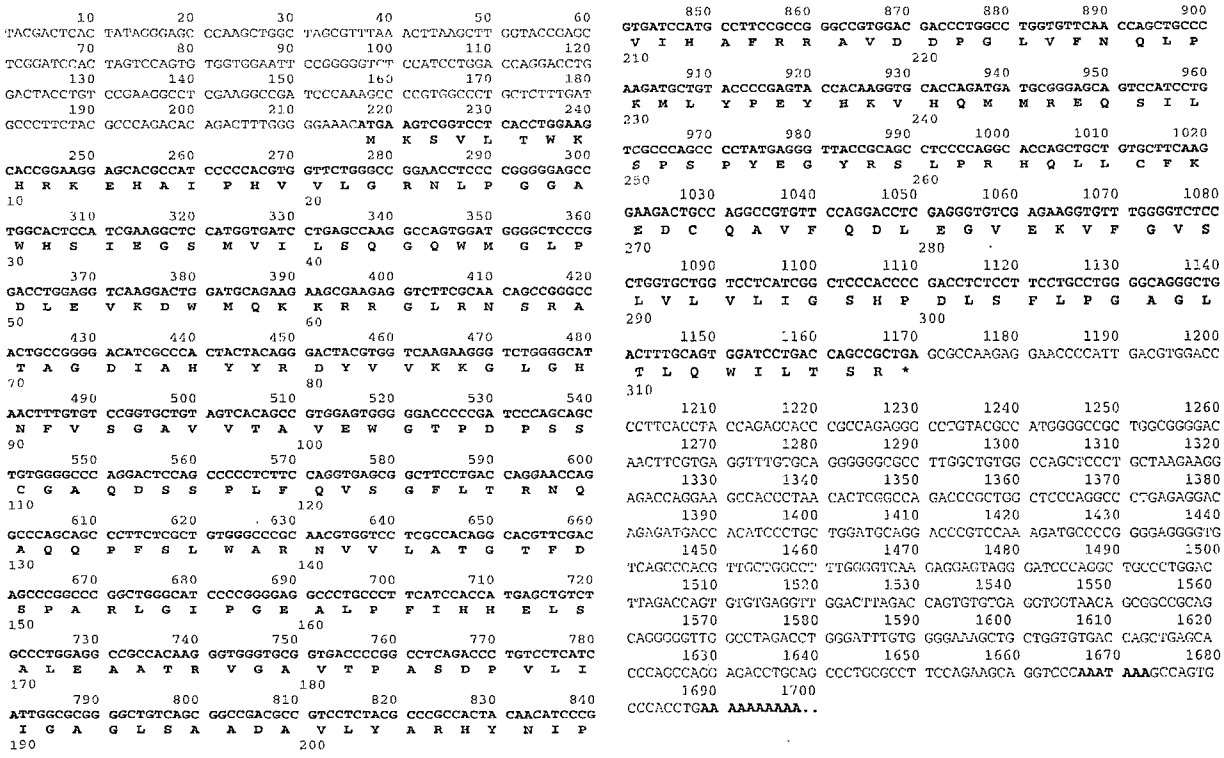


Figure 1. Nucleotide and predicted amino acid sequence of OKL38. The cDNA is 1607 bp and encoded for 317 amino acids with calculated molecular weight of 34.5 kDa.

To determine the distribution of 1.6 kb transcripts, Northern blot analysis was performed using poly "A" RNA derived from various tissues of mature female rats. The band of approximate 1.6 kb were observed in all tissues with the highest levels were seen in the ovary, kidney and liver (Fig. 2). Minor bands of approximate 3.6 and 4.0 kb were also observed.

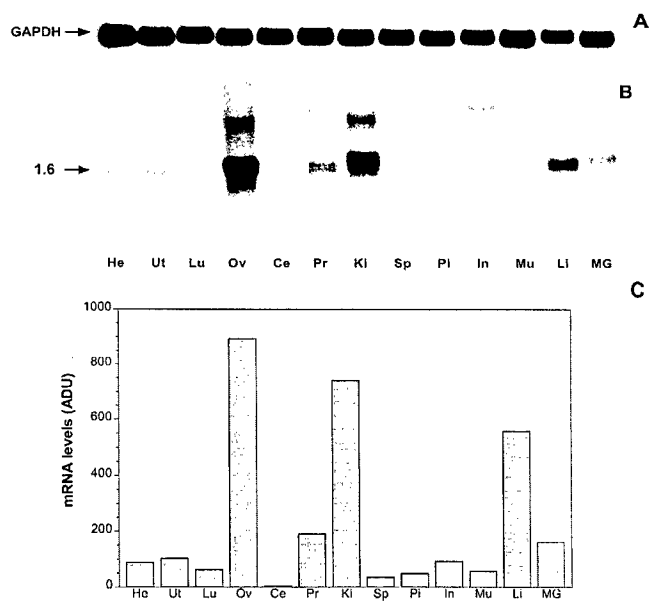


Figure 2. Northern blot analysis of OKL38 gene expression in adult rat tissues. Two μg of poly "A" RNA derived from each tissue of 3 months old rat was subjected to Northern blot analysis. Blots were hybridized with GAPDH (**A**) and human OKL38 (**B**) cDNAs. Densitometric scanning of the 1.6 kb band is shown in (**C**). Tissues are: **He**: heart; **Ut**: uterus; **Lu**: lung; **Ov**: ovary; **Ce**: cerebellum; **Pr**: prostate; **Ki**: kidney; **Sp**: spleen; **Pi**: pituitary; **In**: small intestine; **Mu**: red muscle; **Li**: liver; and **Mg**: mammary gland. Highest levels of OKL38 mRNA were detected in ovary, kidney and liver. High molecular weight transcripts of OKL38 were also detected.

Western blotting using rabbit polyclonal antibody against OKL38 protein recognized a 38 kDa protein in most of the tissues examined with the highest levels found in the heart, cerebellum, kidney and liver (Fig. 3). This protein was thus named as OKL38.

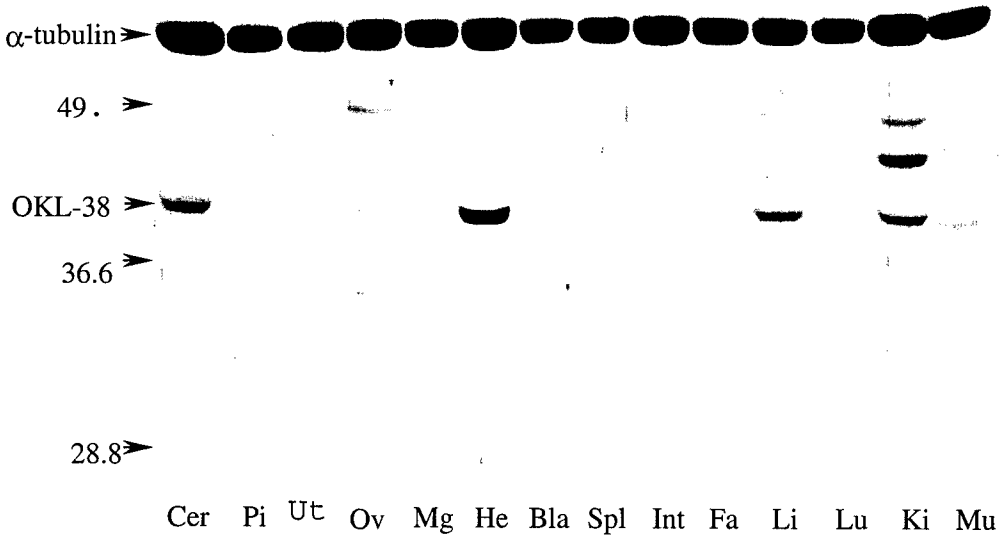


Figure 3. Western blot analysis of OKL38 protein in adult rat tissues. Total proteins extracted from various tissues of 3 months old rat was analysed by Westhern blotting. Blots were incubated with rabbit polyclonal anti OKL38 and mouse anti α-tubulin antibodies. Tissues are: **Cer**: cerebellum; **Pi**: pituitary; **Ut**: uterus; **Ov**: ovary; **Mg**: mammary gland; **He**: heart; **Bla**: bladder; **Spl**: spleen; **Int**: small intestine; **Fa**: fat; **Li**: liver; **Lu**: lung; **Ki**: kidney; **Mu**: red muscle. Highest levels of OKL38 Protein were observed in heart, kidney, liver and cerebellum.

To investigate the changes of OKL38 expression during pregnancy and lactation, poly "A" RNA

and proteins derived from mammary gland at different stages of pregnancy were analysed by Northern and Western blottings, respectively. As shown in figure 4, the levels of OKL38 mRNA were very low in mammary tissue of non-pregnant rats. Following pregnancy, the OKL38 mRNA increased rapidly and maximal OKL38 expression was observed during lactation. Western blot analysis revealed that maximal levels of OKL38 was detected during early pregnancy and remained throughout pregnancy and lactation (Fig. 4D).

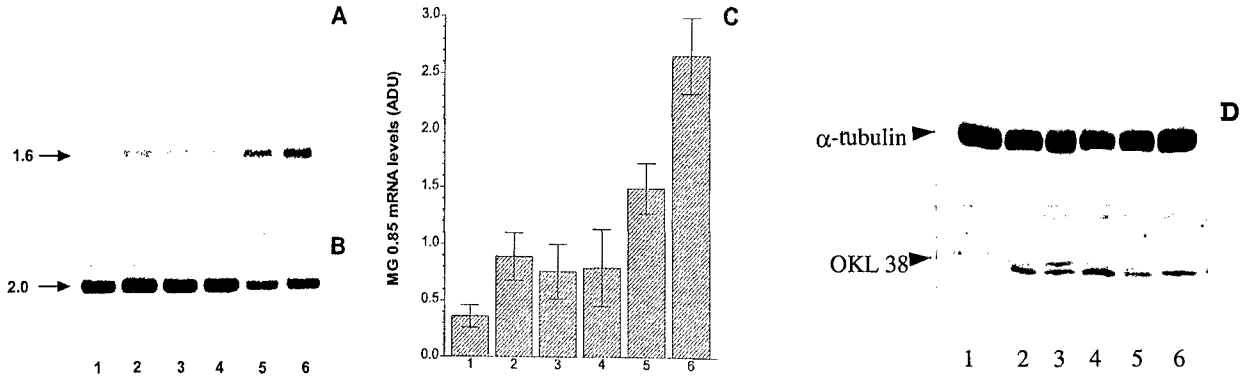
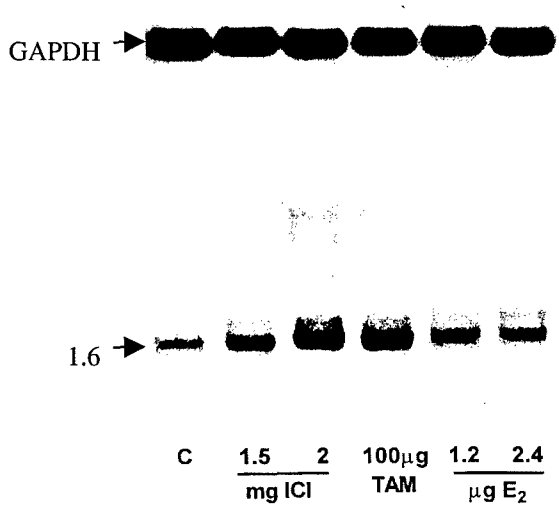


Figure 4. Changes in OKL38 expression in the mammary gland during pregnancy and lactation. Two μg of poly "A" RNA derived from mammary gland on days 0 (Lane **1**), 4 (Lane **2**), 10 (Lane **3**), 16 (Lane **4**), 21 (Lane **5**) of pregnancy and day 3 of lactation (Lane **6**) were subjected to Northern blotting. Blots were hybridized with human OKL38 (**A**) and GAPDH (**B**) cDNAs. Densitometric scanning of the 1.6 kb band is shown in (**C**). OKL38 transcripts increased following pregnancy and lactation. (**D**) Detection of OKL38 protein in the mammary gland during pregnancy and lactation.

To determine if human chorionic gonadotropin (hCG), a known mammary differentiating agent, was capable to increase OKL38 gene expression, female rats were injected with hCG in a manner such as to simulate levels seen at time of lactation (32). Figure 5 shows that hCG induced OKL38 gene expression in a dose-dependent manner.

Figure 5. Effects of human chrorionic gonadotropin on OKL38 gene expression. Female rats were treated with indicated concentrations of hCG for 3 weeks. Two μg of poly "A" RNA derived from mammary tissues were subjected to Northern blotting. Blots were hybridized with human OKL38 (**A**) and GAPDH (**B**) cDNAs. Densitometric scanning of the 1.6 kb band is shown in (**C**). Note that OKL38 gene expression was significantly induced by hCG ($p<0.01$) which is known to induce mammary gland differentiation.



To determine the OKL38 gene expression in human breast cancer cells, poly "A" RNA derived from various cell lines was analysed by Northern blot analysis. Figure 6 shows that although the OKL38 transcripts were detected in all breast cancer cell lines examined, the mRNA levels were about 10 to 15% of that seen in breast tissue. Despite detectable OKL38 mRNA, OKL38 protein in these cell lines was barely detected as determined by Western blotting (data not shown).

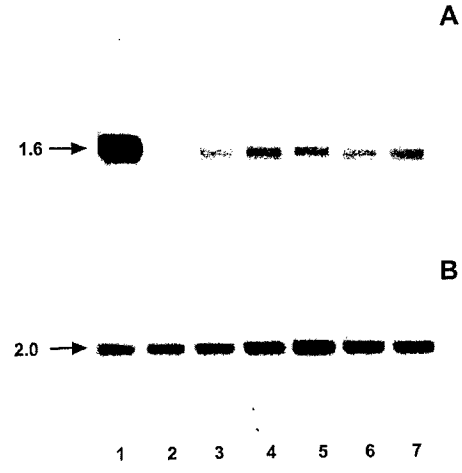


Figure 6. Detection of OKL38 transcripts in human breast cancer cell lines. Two μg of poly "A" RNA derived from human breast cancer cell lines were subjected to Northern blotting. Blots were hybridized with human OKL38 (**A**) and GAPDH (**B**) cDNAs. Positive control mammary gland RNA (Lane **1**) and cell lines: MCF-7 (Lane **2**); T47D (Lane **3**); ZR75 (Lane **4**); MDA-231 (Lane **5**); Hs578T (Lane **6**) and HBL-100 (lane 7). Note that OKL38 gene expression was low in all human breast cancer cell lines.

Since OKL38 is highly expressed in breast tissue during pregnancy, we undertook the first study of OKL38 expression in neoplastic tissue, using the rat DMBA-induced mammary tumour experimental system (33). Figure 7 shows the results of an experiment where we first exposed animals to DMBA, allowed tumours to appear, and then allowed the rats to become pregnant. In lactating rats bearing DMBA-induced tumours, the OKL38 expression seen in normal mammary gland was abundant relative to that seen in most mammary ductal neoplasms. OKL38 proteins shared similar pattern as determined by Western blotting (data not shown).

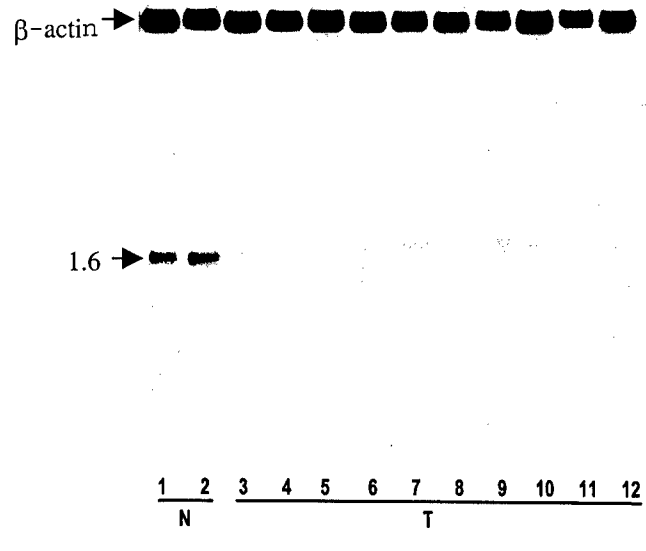


Figure 7. OKL38 gene expression in the mammary gland during pregnancy and DMBA-induced breast tumours. Two μg of poly "A" RNA derived from normal mammary tissues of pregnant rats or DMBA-induced mammary tumours of pregnant rats were analysed by Northern blotting. Blots were hybridized with β-actin (**A**) and OKL38 (**B**) cDNAs. In pregnant rats bearing DMBA-induced tumours, the OKL38 expression seen in normal mammary gland is abundant relative to that seen in all mammary ductal neoplasms.

To test the hypothesis that the gene encoding OKL38 is a tumor suppressor gene, human MCF-7 breast cancer cells were transfected with an expression vector for full-length OKL38 cDNA (pcDNA3.1-OKL38). Figure 8 shows high levels of expression of OKL38 mRNA (~1.6 kb) in representative transfected cell lines SQ13 and SQ18, but the absence of expression in untransfected MCF-7 and the mock-transfected cells. Western blotting with a polyclonal anti-OKL38 antiserum was used to detect OKL38-related protein in the various clones. A 38 kDa protein was detected only in cells transfected with pcDNA3.1-OKL38 (Fig. 8C).

Proliferative behaviour of OKL38-expressing clones was evaluated by determining *cell number* on plastic dishes after 8 days of incubation. The number of cells was significantly less ($p < .05$, Mann-Whitney U-test) in OKL38-expressing transfectants than in controls (Fig. 8D).

To determine if over-expression of OKL38 leads to reduction in tumour formation, *in vivo* tumorgenicity was performed. As shown in figure 8E, the rate of tumor formation following injection of MCF-7 cells was 100% (8 of 8), that of pcDNA3.1-1 mock-transfected cells 88% (7 of 8), and that of SQ13 and SQ18 transfected cells 12.5% (1 of 8) and 25% (2 of 8), respectively.

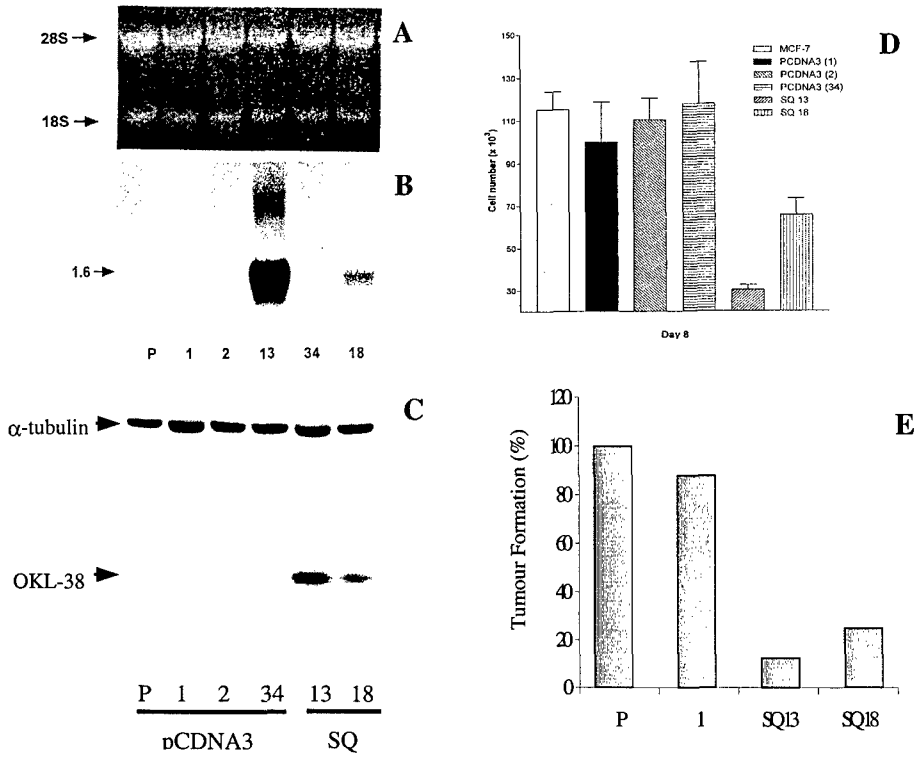


Figure 8. Effects of stable transfection of breast cancer cell line with OKL38 cDNA. Northern blot hybridization with a [^{32}P]dCTP-labeled OKL38 probe (**B**) of total RNA (50 μg/lane) extracted from parental MCF-7 cells (**P**), pcDNA3.1 vector transfected cell lines (lines **1**, **2** and **34**) and OKL38 transfectants (**SQ13** and **SQ18**). RNA loading amounts were compared by ethidium bromide staining of 18S and 28S rRNA (**A**). Immunodetection of 38 kDa OKL38 in stably transfected cell lines SQ13 and SQ18 (**C**). Proliferative behaviour of the clones expressing OKL38 by determining *cell number* on plastic dishes after 8 days incubation (**D**). The number of cells was

significantly less ($p<.05$, Mann-Whitney U-test) in OKL38-expressing transfectants than in controls. Tumour formation of stably transfected cell lines SQ13 and SQ18 (**E**). The rate of tumor formation was significantly less ($p <.05$, Mann-Whitney U-test) in OKL38-expressing transfectants than in controls.

CONCLUSION

Studying breast cancer protection associated with pregnancy, we isolated a pregnancy-induced growth inhibitor cDNA, OKL38, using a method of differential display. Comparison of the nucleotide sequence obtained against the non-redundant nucleotide data base of GeneBank established that OKL38 cDNA was novel and shared no significant homology with any published sequences. The OKL38 cDNA contains 1607 bp and encodes for 317 amino acids with a calculated molecular weight of 34.5 kDa.

The human OKL38 cDNA and its antibody detected OKL38 mRNA and protein from rat and mouse tissues suggesting that OKL38 gene is conserved among human, rat and mouse. In order to determine the homology among rat, human and mouse OKL38 cDNA, experiments are underway to clone the rat and mouse OKL38 cDNAs.

Tissue survey revealed that OKL38 transcripts were detected in all tissues examined with the highest levels in the ovary, kidney and liver. High molecular weight mRNAs were also detected. It is unknown whether these transcripts serves as precursor of mature transcript or different transcripts derived from a result of alternative splicing. Western blot analysis revealed that anti-OKL38 recognized a 38 kDa protein which were abundant in heart, cerebellum, kidney and liver. Despite low levels of OKL38 mRNA in the heart and cerebellum (Figure 2), OKL38 protein content was high in these tissues suggesting that translational and post-translational controls of OKL38 protein present in these tissues.

The gene expression of OKL38 was low in MCF-7 cells and other human breast cancer cell lines examined. Furthermore, OLK38 immunoreactivity is barely detectable in these cells. Increasing OKL38 levels following transfection lead to a reduction in cellular growth and tumour formation in nude mice suggesting OKL38 plays an important role in growth regulation and tumorigenesis. The demonstration of tumour suppressor activity in MCF-7 cells is provocative, but to support the hypothesis that OKL38 is unrelated to characterized growth inhibitory or tumor suppressor protein. The observed tumor suppressor activity of the OKL38 gene is comparable to that previously documented using similar assays for Rb, p53, and H19 (38-41).

The abundance of OKL38 transcripts in poly "A" RNA extracted from the entire mammary gland is low. The expression levels are significantly increased above baseline at the time of the physiological changes associated with pregnancy and lactation (specifically maximal breast epithelial differentiation). *In vivo* experiments shows that hCG-induced OKL38 expression in normal breast tissue. These observations document the existence of hormonal regulation of OKL38 expression. A strong relationship between onset of differentiation, inhibition of proliferation, and onset of OKL38 expression is observed. This observation suggests that a novel approach to breast cancer prevention, at least in certain populations, would therapeutically use hCG to encourage OKL38 expression to

lower breast cancer risk. This approach would in fact represent a mimicking of the well-known protective effect of early pregnancy on subsequent cancer: the terminal differentiation associated with lactation has been proposed as a mechanism underlying the protective effect of pregnancy (32). This information may give insight into novel actions of hCG that may be related to its differentiation and antineoplastic activity (32).

Part 2: Induction of mammary epithelial cell differentiation and inhibition of dimethylbenz(A)anthracene-induced mammary tumour by co-administration of a pure anti-estrogen ICI 182780 and testosterone enanthate.

Control mammary gland had a sparse cluster of epithelial tubules surrounded by a small amount of connective tissue which was in turn embedded in a large fat pad. The epithelial ducts had a narrow, small lumen, lined by cuboidal cells with dark stained nuclei (Fig. 1A). Treatment of intact animals with ICI 182780 resulted in a marked atrophy of the mammary gland. The acinar cells were small and apparently inactive (Fig. 1B). The ICI-induced pattern was characterized by a decreased size of the lobular structures, which consisted of small atrophic alveoli, lined by atrophic and low cuboidal cells. The acinar epithelial cells were apparently inactive, with diminished quantity of cytoplasm (Fig. 1B). Testosterone enanthate stimulated lobuloalveolar and ductal growth, as well as the secretory activity of acinar cells. The lobuloalveolar structures consisted of hypertrophic acinar epithelial cells mainly filled with eosinophilic and, to a lesser degree, clear secretory vacuoles (Fig. 1C). Mammary ducts were only locally, mildly dilated. Co-administration with TE and ICI resulted in a significant increase in lobuloalveolar tissue of the mammary gland (Fig. 1D). The lobular hyperplasia observed was characterized by size and number of the lobular structures. The lobuloalveolar units consisted of groups of alveoli lined by hypertrophic epithelial cells with highly eosinophilic cytoplasm filled mainly with eosinophilic secretory vacuoles similar to that observed in the mammary gland at the end of pregnancy (Fig. 1D).

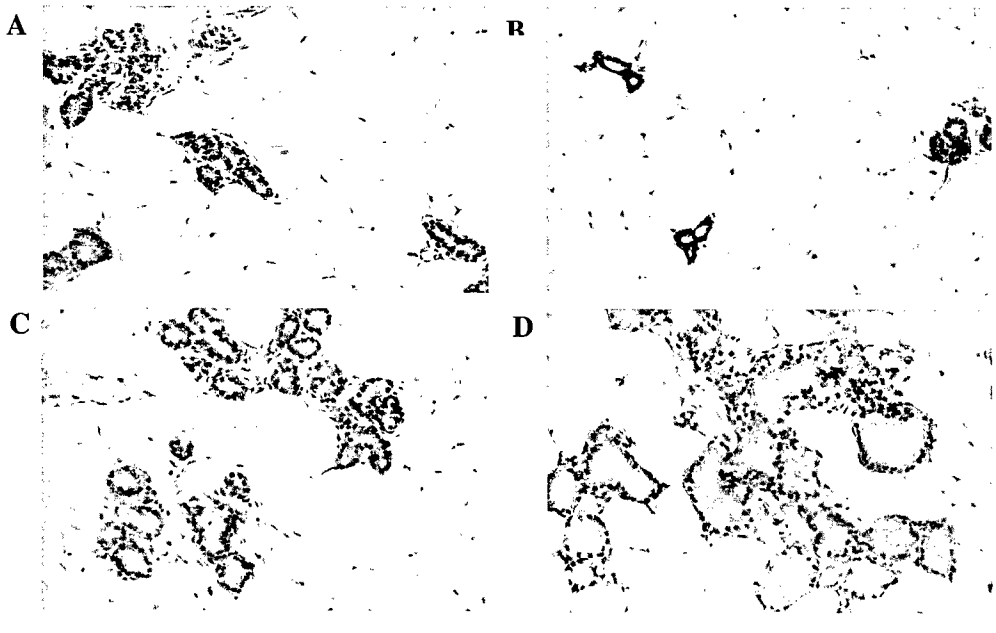


Figure 1. Mammary gland histology in rats treated with vehicles (A), ICI (1 mg/kg/week) (B), TE (1.2 μg/day) (C) or TE plus ICI (D). Note ICI caused marked atrophy of the mammary gland, small atrophic alveoli, lined by atrophic and low cuboidal cells. TE stimulated lobuloalveolar and ductal growth, as well as the secretory activity of acinar cells. TE plus ICI increased lobuloalveolar tissue which consists of groups of alveoli lined by hypertrophic epithelial cells filled with secretory vacuoles similar to that observed in the mammary gland at the end of pregnancy. Haematoxylin-eosine stain was used; magnification. x800.

We determined the effect of hormonal manipulation on Ki-67 expression in mammary tissue. Figure 2 shows the results of an experiment where mammary tissues were collected from rats treated with vehicle, ICI, TE, and ICI plus TE for immunohistochemical analysis of Ki-67 expression. TE significantly increased ($P<0.01$) while ICI significantly decreased ($P<0.01$) the number of epithelial cells expressed Ki-67 as compared to controls. Further reduction in Ki-67 labelling index of the epithelium was seen when ICI and TE were co-administered as compared with the effect of each treatment alone ($P<0.01$).

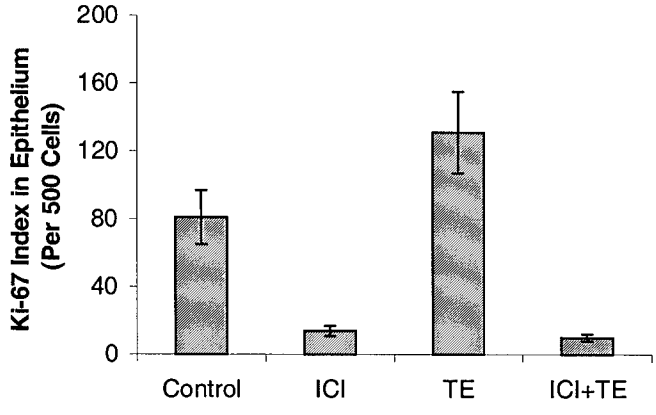


Figure 2. Effects of treatment with ICI (1mg/kg BW/week) or testosterone enanthate (1.2 μg/day) alone or TE plus ICI on proliferation of mammary epithelial cells. ICI either alone or in combination with TE is very effective in blocking Ki-67 indices of epithelial cells. Bars are different letter are significantly different from one another at $P<0.01$). Data are expressed as the mean ± SEM.

Since whey acidic protein (WAP), α-casein, and β-casein and MDGI are hormonally regulated in rat breast epithelial cells and maximally expressed at the time of maximal differentiated function (37), the levels of WAP, α-casein, and β-casein and MDGI transcripts provide good markers for breast differentiation. As shown in Figure 3, the WAP, α-casein and β-casein transcripts were barely detected in control, ICI- and TE- treated mammary glands. Co-administration of TE and ICI resulted in a significant induction in WAP, α-casein and β-casein gene expression.

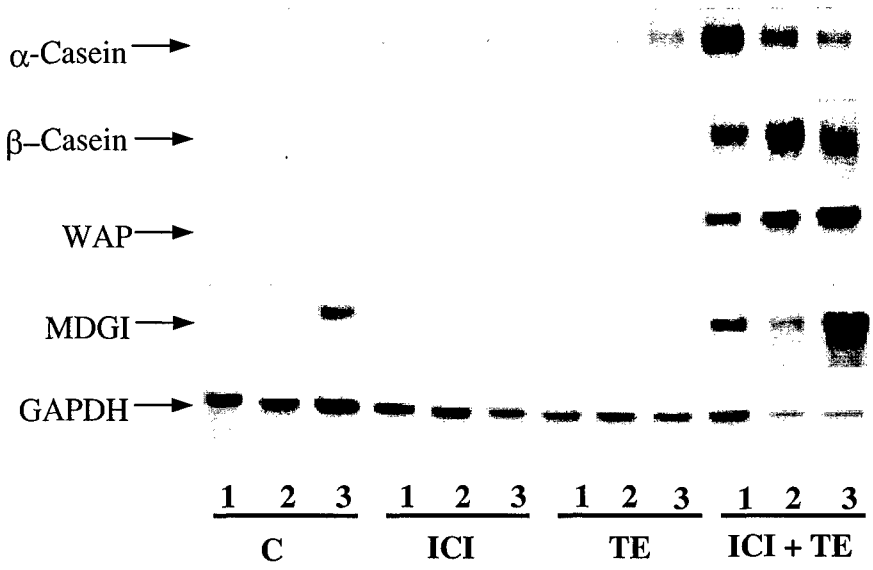


Figure 3. Effects of ICI (1mg/kg BW/week) or testosterone enanthate (1.2 μg/day) alone or in combination on α-casein, β-casein, WAP and MDGI gene expression. Rats were treated with ICI, TE and TE plus ICI as described under Materials and Methods. Total RNA derived from mammary gland was subjected to Northern blotting. Blots were hybridized with rat α-casein, β-casein, WAP, MDGI and GAPDH cDNAs. For each treatment, three representative samples are shown.

To determine whether changes in the mammary gland by TE plus ICI treatment resulted in a decrease in the incidence of carcinogen-induced mammary tumours, the DMBA-induced breast carcinoma in the rat was used. Treatment with ICI significantly decreased tumour incidence from 85% to 40% ($p<0.01$) (Fig. 4A). The average tumour number per tumour-bearing animal and the average tumour area were also significantly reduced ($p<0.01$) (Fig. 4B). TE had no significant effects on tumour incidence, tumour number per animal and tumour size as compared to controls ($P<0.01$) (Fig. 4). Dramatic reduction in tumour incidence, tumour number and tumour size was found in the animals who received both ICI and TE ($p<0.01$) (Fig. 4).

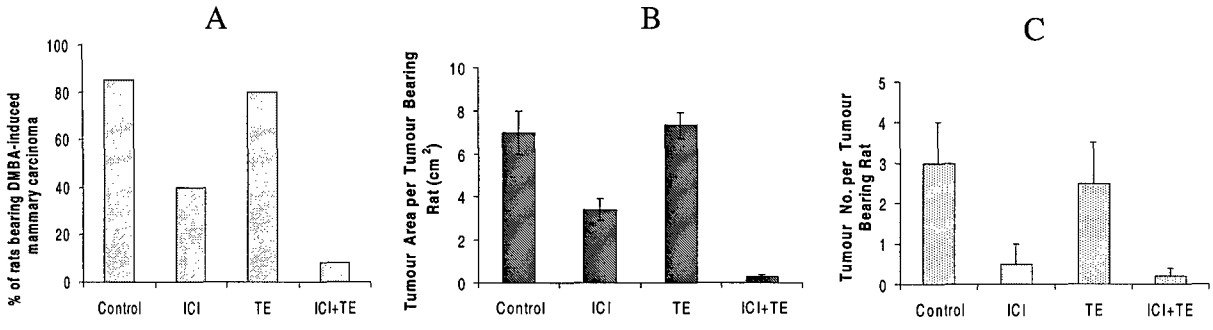


Figure 4. Effects of treatment with ICI (1mg/kg BW/week) or testosterone enanthate (1.2 μg/day) alone or in combination for 150 days on (A) the incidence of DMBA-induced mammary carcinoma, (B) on average tumour number per tumour-bearing animals, and (C) on average tumour size per tumour-bearing rat. Note that ICI treatment caused a significant reduction in tumour incidence, tumour number and tumour size as compared to vehicle and TE treatments. TE plus ICI further decreased the above parameters. Bars in different letter are significantly different from one another at $P<0.01$). Data are expressed as the mean ± SEM.

CONCLUSION

The Ki-67 labelling index of the epithelium was significantly reduced upon ICI treatment. The reduction in this proliferative marker was accompanied by a significant reduction in epithelial cells. Treatment with ICI induces atrophy of the mammary gland. Testosterone enanthate increased the Ki-67 labelling index and stimulated the ductal and the lobular structure. Mammary epithelial cells became terminally differentiated when both ICI and TE were coadministered. Morphological differentiation of epithelial cells in ICI and TE treated glands was quite similar to that observed at the end of pregnancy and during lactation. ICI when given with TE blocked TE-induced Ki-67 expression, induced whey acidic protein, α-casein and β-casein gene expression. In addition, mammary-derived growth inhibitor MDGI gene expression was greatly induced when both ICI and TE were coadministered. Since WAP, α-casein, β-casein and MDGI genes are preferentially expressed by terminally differentiated mammary epithelial cells (37), the presence of their transcripts provide a good marker for breast differentiation.

Using the rat DMBA-induced breast carcinoma, we demonstrated that ICI could effectively decrease tumour incidence, the average tumour number per tumour-bearing animal and the average tumour area. TE had no significant effects on tumour incidence, tumour number per animal and tumour size as compared to controls. Dramatic reduction in tumour incidence, tumour number and tumour size was found in the animals who received both ICI and TE suggesting that ICI and testosterone enanthate exerted more potent inhibitory effects than each compound used alone on the development of DMBA-induced rat mammary carcinoma.

SO WHAT

In developed countries, reproductive behaviour is determined by both social and economic forces. Thus, for educational, career-related and other reasons, millions of women in these countries are delaying childbearing and having fewer children, in general, than their mother and grandmothers did. Unfortunately, such life decisions will lead to higher rates of breast and ovarian cancer. It is impractical to suggest early pregnancy as a breast cancer prevention strategy. Therefore, a novel approach to breast cancer prevention, at least in certain populations, using physiological signals that encourage terminal differentiation to lower breast cancer risk was needed. The above described data clearly demonstrate the additive chemopreventive effects of the antiestrogen ICI 182780 and testosterone enanthate on the development of mammary carcinoma as well as differentiative effects of such a combination on mammary epithelial cells. This hormonal regime, when given to women in their teens or early twenties, stimulates mammary differentiation and could potentially reduce the risks for breast cancer. This approach would in fact represent a mimicking of the well-known protective effect of early pregnancy on subsequent breast cancer (5). This strategy is a more physiological approach than long-term administration of tamoxifen, as it seeks to mimic the physiology associated with reproductive behaviour that naturally reduces breast cancer risk. Although our data demonstrate that co-administration of ICI 182780 and testosterone enanthate to rats can differentiate mammary epithelial cells, clinical trials are further needed to clarify the effects of these drugs in human.

STATEMENT OF WORK

To date we have completed Task 1-5 of the Statement of Work outlined in the proposal: Purification of UDGI from rat uterine fluid. In doing so we came across some unanticipated problems:

The differences between the rat 1.1 and 1.4 kb UDGI transcripts were the length of the 5' untranslated region. Comparison of the nucleotide sequence against the non-redundant nucleotide database of Genebank established that the UDGI was ps20. Blast search revealed more than 99% homology with rat ps20 sequence (42) (Refer to Appendix 1).

Since rat ps20 (42) and human ps20 (43) cDNA have been cloned and reported, we decided to change research topic and focused more on the cloning and characterization of a novel pregnancy-induced growth inhibitor in mammary gland (Appendix 8). During the course of cloning and characterization, we also identified a hormonal regime, when given to female rats (Appendix 9), stimulated mammary differentiation and inhibited dimethylbenz(A)anthracene-induced mammary carcinoma.

Key Research Accomplishments:

- Purification of rat ps20 protein, cloning rat ps20 from rat uterine cdNA library, generating rat ps20 antibody, examining hormonal regulation of ps20 in rat mammary gland and tumours and transfecting ps20 into breast cancer cells (Appendix 1).

- Cloning and characterization of a novel pregnancy-induced growth inhibitor in mammary gland (Appendix 8).

- Identifying a hormonal regime (a pure anti-estrogen ICI 182780 plus testosterone enanthate) that stimulates mammary differentiation and inhibited dimethylbenz(A)anthracene-induced mammary carcinoma (Appendix 9).

Reportable Outcomes:

Personnels receiving pay from research effort: (90% support by DAMD17-97-1-7084).

Daniel Song (post-doctoral), Xiaoming Deng (technician), Alex Codrington (technician) and Daniella Marcantonio (Ph.D student).

Abstracts: (Degree of support: 100%)

1. Huynh, H. Inhibition of insulin-like-growth factor signaling pathways in mammary gland a pure anti-estrogen ICI 182780. AACR, San Francisco, CA. 2000. (100% support by DAMD17-97-1-7084).). See Appendix **2**.

2. Huynh, H. Cloning and characterization of a novel pregnancy-induced growth inhibitor in the mammary gland. Department of Defense Breast Cancer Research Program Meeting: Era of Hope, Atlanta, Georgia. June 8-12, 2000. (100% support by DAMD17-97-1-7084). See Appendix **3**.

3. Huynh, H. Androgen Sensitive Gene (ASG), a novel gene associated with cell proliferation and is regulated by steroid hormones. ENDO, San Diego, CA. 1999. (100% support by DAMD17-97-1-7084). See Appendix **4**.

Patents applied for:

1. Huynh, H. (2000). Cloning and characterization of a novel pregnancy-induced growth inhibitor in the mammary gland. (Patent filed ref no: 2269060/VPA/SJW). (80% support by DAMD17-97-1-7084).). See Appendix **5**.

2. Huynh, H. (2000). Induction of mammary epithelial cell differentiation and inhibition of dimethylbenz(A)anthracene-induced mammary tumour by co-administration of a pure anti-estrogen ICI 182780 and testosterone enanthate. (Patent filed ref no: 2269349/VPA/aal). (70% support by DAMD17-97-1-7084).). See Appendix **6**.

3. Huynh, H. (2000). Characterization of a novel tamoxifen-induced cDNA (UO-44) in the rat uterus and ovary. (Patent filed ref no: 2269336/EJH/aal). (70% support by DAMD17-97-1-7084).). See Appendix **7**.

4. Huynh, H. (2000). Co-administration of finasteride and the pure anti-estrogen ICI 182780 acts synergistically in modulating IGF-system and PSA in rat prostate. (Patent applied in process:). (75% support by DAMD17-97-1-7084).

Publications:

1. Huynh, H. (2000). Cloning and characterization of a novel pregnancy induced growth inhibitor in the mammary gland. Cancer Research (subsmitted). (90% support by DAMD17-97-1-7084). See Appendix **8**.

2. Huynh, H. (2000). Induction of mammary epithelial cell differentiation and inhibition of dimethylbenz(A)anthracene-induced mammary tumour by co-administration of a pure anti-estrogen ICI 182780 and testosterone enanthate. Cancer Research (submitted).(90% support by DAMD17-97-1-7084). See Appendix **9**.

3. Huynh, H. (2000). Inhibition of insulin-like-growth factor signaling pathways in mammary gland a pure anti-estrogen ICI 182780. Cancer Research (submitted). (75% support by DAMD17-97-1-7084). See Appendix **10**.

4. Huynh, H. Chan, T.W., Fouti, N., and Ng. C.Y. (2000). Characterization of a novel tamoxifen-induced cDNA (UO-44) in the rat uterus and ovary. Journal of Biological Chemistry (submitted). (90% support by DAMD17-97-1-7084). See Appendix **11**.

5. Huynh, H., Alpert, L., Alauoi-Jamali, M., Chan, T.W. Mark., and Ng, C.Y. (2000). Co-administration of finasteride and the pure anti-estrogen ICI 182780 acts synergistically in modulating IGF-system and PSA in rat prostate. Cancer Research (submitted). (75% support by DAMD17-97-1-7084). See Appendix **12**.

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Appendices

1 Annual Report for DAMD17-97-1-7084 (June 1999)

Abstract:

2 Inhibition of insulin-like growth factor signaling pathways in mammary gland by pure anti-estrogen ICI 182780

3 Cloning and characterization of a novel pregnancy induced growth inhibitor

4 Androgen sensitive gene (ASG), a novel gene associated with cell proliferation and is regulated by steroid hormones.

Patent pending:

5 Novel Polynucleotide and polypeptide and uses therefor

6 Novel Compositions and methods of using them

7 A nucleic acid molecule and uses therefor

Publication:

8 Cloning and charcterization of a novel pregnancy-induced growth inhibitor in mammary gland

9 Induction of mammary epithelial cell differentiation and inhibition of dimethylbenz(A)anthracene-induced mammary tumour by co-administration of a pure anti-estrogen ICI 182780 and testosterone enanthate

10 Inhibition of insulin-like growth factor signaling pathways in mammary gland by pure anti-estrogen ICI 182780

11 Characterization of a novel tamoxifen-induced cDNA (Uo-44) in the rat uterus and ovary

12 Co-administration of finasteride and the pre anti-estrogen ICI 182780 acts synergistically in modulating the IGF-system and the PSA in rat prostate

13 Curriculum vitae for Hung The Huynh

Appendix 1

AD_____

GRANT NUMBER DAMD17-97-1-7084

TITLE: Characterization of Uterine Derived Growth Inhibitor: A Novel Growth Inhibitor of Estrogen Receptor

PRINCIPAL INVESTIGATOR: Hung T. Huynh, Ph.D.

CONTRACTING ORGANIZATION: McGill University
Montreal, Quebec, Canada H3T 1E2

REPORT DATE: June 1999

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
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13. ABSTRACT *(Maximum 200 words)*

We reported the purification of UDGI, a uterine derived growth inhibitor. UDGI protein shares 98% identity to ps20 growth inhibitor isolated from urogenital sinus mesenchymal cells. Although purified UDGI potently inhibited breast cancer cell growth, recombinant UDGI exhibited mild inhibition. UDGI antibody recognized native UDGI from uterine fluid and conditioned media of UDGI-transfectant cells with molecular mass of 24, 27 and 29 kDa. UDGI cDNA was isolated from a rat uterus cDNA library. UDGI transcripts were detected in rat mammary tissue and in various female rat tissues with highest expression in the lung and heart. *In vitro*, UDGI secretion was reduced by IGF-I, IGF-II, insulin and TGF-α. *In vivo*, UDGI expression was inhibited by estradiol and induced by pure antiestrogen ICI 182780 (ICI) and tamoxifen. UDGI mRNA was barely detected in mammary tissue of old female rats and in DMBA mammary tumours. Suppression of tumour growth by ICI and tamoxifen was associated with upregulation of UDGI gene expression. Acceleration of apoptosis was observed in stable UDGI-transfectant MCF-7 cell lines following serum starvation. The results suggest that UDGI may function as a mediator of local growth and apoptosis mechanisms.

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Table of Contents

PAGE

INTRODUCTION

I. BREAST CANCER

Breast cancer is the most common form of cancer among non-smoking women in the Western society. Approximately one in nine women in North America living to age 80 will develop breast cancer (1). The incidence of breast cancer appears to be increasing with an annual worldwide rate of over one million predicted cases by the turn of this century (2). This high occurrence of metastatic breast cancer is a major challenge, particularly as the probability of survival beyond 5 years is low for patients with metastatic disease.

Breast cancer is one of a small number of malignancies in which both genesis and growth have been linked to hormonal factors. About one-third of all metastatic human breast cancers is responsive to existing endocrine therapies (3). Antiestrogens inhibit the proliferation of *in vitro* human breast cancer cells expressing the estrogen receptor (ER), and have proven to be clinically useful for ER positive tumours (4-7). However, the efficacy of current hormonal therapies is restricted by the progressing resistance of an overwhelming majority of tumours. It is often noted that during the course of hormonal therapy, ER positive breast cancer cells lose their dependence on estrogens, their ability to express ER, and their responsiveness to endocrine therapies (1).

Growth factors comprise a key component of stromal-epithelial interactions. Growth factors that have been implicated in mammary gland biology include: the FGF family, TGF-ß family, TGF-α, the insulin-like growth factor family, EGF, heparin-binding EGF, TNF-α, and heparin growth factor-scatter factor (reviewed in (8). Each of these growth factors are bound to the extracellular matrix, cell membrane, or other binding proteins in an inactive or sequestered form and their activity is regulated by secreted proteases and protease inhibitors. The actions of proteases and protease inhibitors are fundamental to tissue homeostasis through the regulation of both growth factor bioavailability and cell interaction with the extracellular matrix. Alterations in protease and protease inhibitor actions affect tissue development patterns, angiogenesis, cell motility, and tumour invasion (9-12).

II. UTERINE SECRETION FUNCTIONS

It is clear that the female genital tract, including the uterus, exhibits considerable control over the ability of a conceptus to develop (13,14). The uterus expresses and secretes a number of growth factors (15-20) and other regulatory polypeptides (19,21) in response to ovarian steroid hormones. These polypeptides are thought to play a part in directing or limiting the growth and development of the uterus.

Although several growth inhibitors have been identified over the last few years, there is no reason for us to believe that we have identified them all. Our previous work examining the molecular basis for the association of estradiol and tamoxifen with endometrial carcinoma (19,22) showed

the inhibitory effects of uterine fluid (UF) on breast cancer cell growth. We hypothesized that the uterus synthesizes and secretes a growth inhibitor to limit the proliferative response to estradiol stimulation. This led to our interest in characterizing the growth inhibitory activity of UF.

EXPERIMENTAL PROCEDURES

The following products were used to carry out the experiments: Bio-Gel P-100 (100-200 mesh) and P-30 (100-200 mesh) chromatography gels, glycine, and acrylamide from Bio-Rad; diethylaminoethyl cellulose (DE-52) ion exchange resin and glass fiber filters (934-AH, 2.4 cm) from Whatman; dialysis tubing no. 3 (M_r cutoff = 3,500) from Spectrapore (Los Angeles, CA); TCA, acetonitrile and formic acid from Pharmacia; tissue culture media and fetal calf serum from Gibco; 96-well plates from Nunc; and [^{3}H]-thymidine from ICN.

Uterine fluid collection. Silastic tubes containing 17-ß estradiol were implanted into the back of 50 day-old ovariectomized rats. Two weeks after estradiol implantation, the animals were sacrificed with carbon dioxide. The UF was removed from both uteri and centrifuged at 14000 g at 4^{0}C for 30 min to remove cellular debris. The supernatant was stored at -75^{0}C until analysis.

Cell Proliferation Assays. Cell proliferation was determined by evaluating [^{3}H]-thymidine incorporation. For the [^{3}H]-thymidine incorporation assay, the cells were seeded at 1.0 x 10^4 cell/well in 96-well plates and allowed to attach for 24 h. The cells received a 50μl aliquot of the sample to be tested (previously vacuum-dried and resolubilized in 60 μl growth medium in sterile conditions) and were incubated for an additional 24 h. The cultures were pulsed with [^{3}H]-thymidine (2 μCi/ml) during the final 2 h of incubation. The assay was terminated by fixing the cell monolayers *in situ* with 10% TCA (250 μl/well) for 2 h at 4^{0}C, washing with 100% methanol (5 min, RT) followed by 2 washes with distilled water. The plates were then allowed to air-dry for 10 min. The monolayers were hydrolysed with 1 N NaOH (200 μl/well, 5 min, 50 ^{0}C), and 150 μl aliquots were added to scintillation vials containing 150 μl of 1 N HCl to neutralize the pH. The radioactivity was determine by scintillation counting and the results are presented as the reciprocal of incorporated counts/min to reflect the inhibition of [^{3}H]-thymidine incorporation as a peak of activity.

Ion exchange chromatography. UF was filtered through a 0.45 μm filter and applied directly to a DE-52 anion exchange column (1.5 x 9.5 cm, hydrostatic pressure = 30 cm) equilibrated in 20 mM of ammonium carbonate buffer (pH 8.85). The column was washed with 3 bed volumes of the same buffer, eluted with a linear gradient of ammonium carbonate (20-300 mM, pH 8.9, 150 ml) and 5 ml/fraction were collected. The absorbance at 280 nM (A_{280}) and conductivity were determined for each fraction. 30 μl aliquots of each fraction were directly vacuum-dried in sterile microfuge tubes and resolubilized in 100 μl of sterile growth medium. 60 μl aliquots/well were assayed directly for Hs578T cell growth activity by evaluating [^{3}H]-thymidine incorporation.

Gel filtration chromatography. To assign a protein species to a peak of biological activity, 500 μl of UF were dialysed against 1 M acetic acid (pH 2.25) and lyophilized. The lyophilized proteins were redissolved in 1 M acetic acid (1ml) and chromatographed through a Bio-Gel 200 gel filtration column (2.5 x 70 cm), equilibrated in 1 M acetic acid by gravity flow (45 cm), and 3 ml fractions were collected. 100 μl aliquots were vacuum-dried and resolubilized in 65 μl of growth medium. 60 μl were added to Hs578T cells for [^{3}H]-thymidine incorporation assay.

For additional studies and purification of UDGI on a large scale, UF was processed through the ion exchange chromatography step. Pooled fractions from biologically active peaks were prepared by dialysis (Spectrapore no. 3 tubing, 3,500 M_r cutoff) against 1 M acetic acid (pH 2.25) overnight at 4°C. Dialysed samples were frozen, lyophilized and stored at -20°C. The lyophilized samples were solubilized in 1 M acetic acid (1 ml) and applied to a Bio-Gel P-100 column (1.4 x 70 cm), equilibrated in 1 M acetic acid (pH 2.25). The proteins were eluted with a hydrostatic pressure of 55 cm, and 1.4 ml fractions were collected. 100 μl aliquots from each fraction were vacuum-dried in sterile microcentrifuge tubes and resolubilized in 65 μl of growth medium. 60 μl were added to Hs578T cells for [^{3}H]-thymidine incorporation assay.

Reverse phase high performance liquid chromatography. All samples for HPLC analysis were first processed through ion exchange chromatography and gel filtration chromatography. Samples (pooled fractions from bioactivity peak) were either vacuum-dried or lyophilized and resolubilzed in 50% formic acid (0.5 ml). Samples were applied (3 consecutive 150 μl applications) to a Waters C-18 reverse phase column fitted to an HPLC system composed of a Waters 712 WISP automatic injector, a Beckman 412 controller, 110 A pumps, and 165 variable wavelength detector. The proteins were eluted with a 1.0 ml/min/fraction flow rate with 0.1% trifluoroacetic acid in a linear acetonitrile gradient. 100 μl aliquots from each fraction were vacuum-dried in sterile microcentrifuge tubes and resolubilized in 65 μl of growth medium. 60 μl were added to Hs578T cells for [^{3}H]-thymidine incorporation assay.

SDS-polyacrylamide gel electrophoresis (PAGE). Samples were vacuum-dried, resolubilized in Laemmli sample buffer and analysed by SDS-PAGE following the procedures of Laemmli (23).

Protein sequencing: Approximately 1-2 μg of purified UDGI proteins were electrophoresed through SDS-PAGE gels in the buffer containing 150 mM thyoglycolic acid at 45 volts to reduce amino-terminal blockage. The gels were calibrated with transfer buffer (35 mM Tris-base, 192 mM glycine) for 30 min and assembled with Pro-Blot polyvinylidene difluoride (PVDF) membranes in a Bio-Rad Trans-Blot cell apparatus. Proteins were electrobotted using 27 V for 16 h at room temperature. Proteins were visualized by Coomassie staining, the UDGI protein bands were excised, and the sequence was determined using Applied Biosystems 477A and 773A protein sequencers.

DNA sequencing: Multiple pass DNA sequencing was performed on an Applied Biosystem model 377 Sequencer version 2.1.1 (Seldon Biotechnology, McGill University) using Ampli-Tag polymerase and UDGI specific primers and double-strand DNA. Sequences were assembled using MacVector version 1 and AssemblyLign version 1.0 (Kodax).

Sequence Analysis: Nucleotide sequence searches were performed on available data bases using the BLASTN and TBLASTN (blast enhanced alignment utility) algorithms.

Preparation of UDGI antisera: For initial immunization, purified UDGI was solubilized in sterile distilled water (500 μg/ml), mixed with Freund's complete adjuvant (1:1 v/v) and injected in 1 ml (250 μg) aliquot at multiple subcutaneous sites of two female New Zealand rabbits. At 3 weeks following immunization, each rabbit received a booster of 100 μg of peptide in Freund's incomplete adjuvant injected subcutaneously. The boosting was repeated one every 5 weeks. Serum was collected every 2 weeks after the third boost and was tested for UDGI specific

antibody. High titer anti-serum was obtained after the fifth boost and was used to detect UDGI.

MCF-7 cell stable transfectant cell lines. The entire coding region of UDGI cDNA was cloned into mammalian expression vector pcDNA3.1 (Invitrogen) to create the UDGI-pcDNA3.1 expressing vector. The UDGI-pcDNA3.1 sequence was confirmed by sequencing. MCF-7 cells were seeded at 2x 10^5 in 100 mm culture dishes in 90% α-MEM (Life Technologies, Inc.) containing 10% FCS with Garamycine 24h prior to transfection. Cells were transfected with 5 μg of UDGI-pDNA3.1 DNA or pDNA3.1 control plasmid DNA and 28 μl of Lipofectamine reagent (Life Technologies) following manufacturer recommendations. 48h following transfection, cells were split 1:10 and replaced with growth medium containing 800 μg/ml G418 (Calbiochem, La Jolla, CA). After 4 weeks, clones were isolated, expanded and assayed for UDGI expression by western blot analysis in conditioned medium.

Expression and purification of UDGI-His. The coding region of UDGI without the signal peptide, was cloned into pQE-30 bacterial expression vector (Qiagen), which allows the incorporation of a His-Tag at the carboxyl terminus. The UDGI-QE containing clone was confirmed by sequencing and transformed into the M15 bacterial expression cell line (Qiagen). 500 ml cultures were grown and induced by 1 mM isopropyl-1-thio-beta-D-galactopyranoside for 2h, and soluble cell extract generated by resuspending in buffer containing 8 M urea, 0.1 M NaH_2PO_4 and 0.01 M Tris.Cl, pH 8.0 for 30 min followed by centrifugation at 13000 rpm for 30 min. Soluble recombinant UDGI His-tagged protein was purified on a nickel-charged resin (Qiagen). The column was washed 3 times with buffer containing 8 M urea, 0.1 M NaH_2PO_4 and 0.01 M Tris.Cl, pH 6.3 and eluted with buffer containing 8 M urea, 0.1 M NaH_2PO_4 and 0.01 M Tris.Cl, pH 4.5 as recommended by the manufacturer. Collected fractions were analysed by SDS-PAGE. Fractions containing proteins were pooled and dialysed against 1 M of acetic acid (pH 2.5) overnight at 4°C. The dialysed samples were quickly frozen, lyophilized, and stored at -20 °C.

Western Analysis. UDGI-transfectant MCF-7 cells and primary mammary cells were grown in growth medium. Both were switched to serum-free medium containing appropriate treatment. Conditioned medium was collected every 24 or 48 hours, clarified by centrifugation, and stored at -20 °C until analysis. Western analysis was performed as described (24). Blots were incubated with rabbit anti-UDGI antibody (1:2500) and horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody (1:7500). Blots were visualized with a chemiluminescent detection system (ECL, Amersham) and exposed to film for 10 sec to 45 sec.

UDGI expression in mammary tissues of 2 month-old rats, 14 month-old rats, in DMBA mammary tumour and following estradiol and anti-estrogen treatments. Animal experiments were approved by McGill University Animal Care Committee. Intact female Sprague-Dawley female, either 60 days old or 14 months old at the beginning of the experiments, were obtained from Charles River, Quebec.

To study effect of estradiol on UDGI gene expression. 0.5 cm, 1.0 cm and 1.5 cm silastic tubes (0.04 in. ID, Dow corning, Michigan) containing 17-ß oestradiol were implanted into the back of the neck of different groups of rats. Control rats were experienced the same surgical implantation with empty silastic tubes. Based on previous published work (25), the release rate of 17 ß-oestradiol from silastic implants was documented to be 2.4 μg/cm/day.

To examine the effects of tamoxifen on UDGI gene expression, rats were implanted with either 2 cm or 4 cm silastic tubes (0.12 in. ID, Dow corning, Michigan) containing tamoxifen (Sigma) were implanted into the back of the neck of different groups of rats. The release rate was 25 μg/cm per day.

To investigate the effects of ICI 182780 on UDGI gene expression, 2 month-old rats were injected with 1 mg, 1.5 mg, and 2 mg/kg BW ICI 182780 (Zeneca Pharmaceuticals) in castor oil per week for 3 weeks. Control rats received only castor oil. To induce UDGI expression in mammary gland of old rats, 14 month-old rats were injected with either castor oil or 2 mg/kg BW ICI 182780.

To study the UDGI expression in mammary tumours and the ability of antiestrogens to induce UDGI expression in DMBA breast tumours. The DMBA tumour model was employed (26). Rats bearing DMBA tumours were treated with either vehicle or 1 mg ICI/kg BW or 5 mg tamoxifen/kg BW. Antineoplastic activity was quantitated as mean tumour burden (mm^3) in treated animals after 2 weeks.

At the end of the experiment, animals were sacrificed using carbon dioxide at the end of the experiment. The mammary tissues or tumours were excised, trimmed and frozen in liquid nitrogen and stored at -70 ^{0}C for RNA extraction. Part of the mammary tissue was fixed in 10% buffered formalin for histochemical studies.

Detection of apoptosis: Five μM sections of mammary tissue were used to detect apoptotic cells using TUNEL assay. Fragmented DNA was labelled using the ApoAlert DNA fragmentation assay (Clontech Laboratories, Palo Alto, CA) as described by the manufacturer.

Northern analysis of UDGI gene expression in mature female rat tissues. Total RNA was isolated from tissues and DMBA tumours using RNAZol-B (Teltest, TX). Northern Blots were performed as described (27). Blots were hybridized either with UDGI or ß-actin (ATCC) or GAPDH cDNA inserts.

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RESULTS

We undertook the first study of the antiproliferative effect of UF *in vitro,* using rat primary endometrial cells, non-mammary cell lines and variety of human and bovine, transformed and non-transformed mammary epithelial cell lines. Figure 1 shows the effects of UF on [^{3}H]-thymidine incorporation into various cell lines and primary uterine cells. The addition of 1% of UF to the culture for 24h resulted in a marked inhibition of proliferation of the breast cancer cell lines. The inhibition was very pronounced for the ER negative cell lines (HH2a, Hs578T, MDA 231, and 16HH2a). The ER positive T47D cell line was distinctly inhibited by UF as well (Fig. 1A). The human HBL-100 and BT-20 cell lines were not inhibited by UF (Fig. 1A).

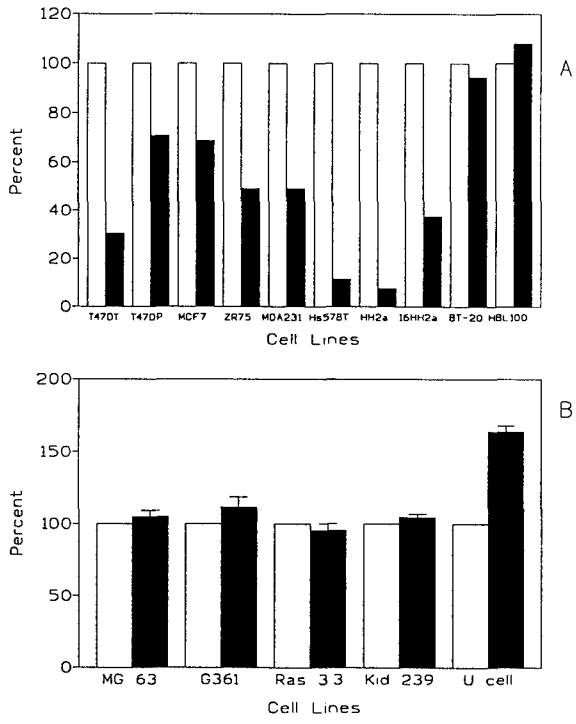

Figure 1. Effect of uterine fluid (UF) on mammary cell (A) and non-mammary cell (B) DNA synthesis. Confluent cultures of target cells for inhibition were trypsinized and plated at 2.5 x 10^4 cells in 24-plate multi-well dishes in 2.5% FCS. After 48h, the cell monolayer was rinsed twice with phenol-red serum free (PSF) media and incubated for 24h in PSF media containing 2.5% double charcoal stripped serum. After 24h, the cells were washed twice with PSF media and then incubated in triplicate without (white bars) or with (black bars) 10 µl/ml of UF added to the PSF media for 24h. At the end of incubation, [^{3}H]-thymidine incorporation into DNA was determined as described (28). The experiments were repeated 3 times with similar results and the means of triplicate determinations were plotted as percent of control.

This inhibition was very specific for breast epithelial cells, since the UF enhanced DNA synthesis in rat primary uterine cells (U cell, Fig 1B). The inhibition of the rat UF was not species-specific, since inhibition of bovine and human breast epithelial cell lines was observed. Furthermore, UF had neither stimulatory nor inhibitory effects on human melanoma (M 361), human osteosarcoma (MG 63), mouse, or rat intestinal epithelial (Ras 3.3) and human kidney epithelial (Kid 239) cell lines (Fig. 1B).

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To study the dose-response of human breast cancer cells to UF, MCF-7, Hs578T and MDA 231 cells were cultured as described above. Various concentrations of UF were added to the cultures and the incubations continued for another 24h. [^{3}H]-thymidine incorporation into DNA was determined as described above. As shown in figure 2, UF inhibited DNA synthesis in three cell lines in a dose-dependent manner.

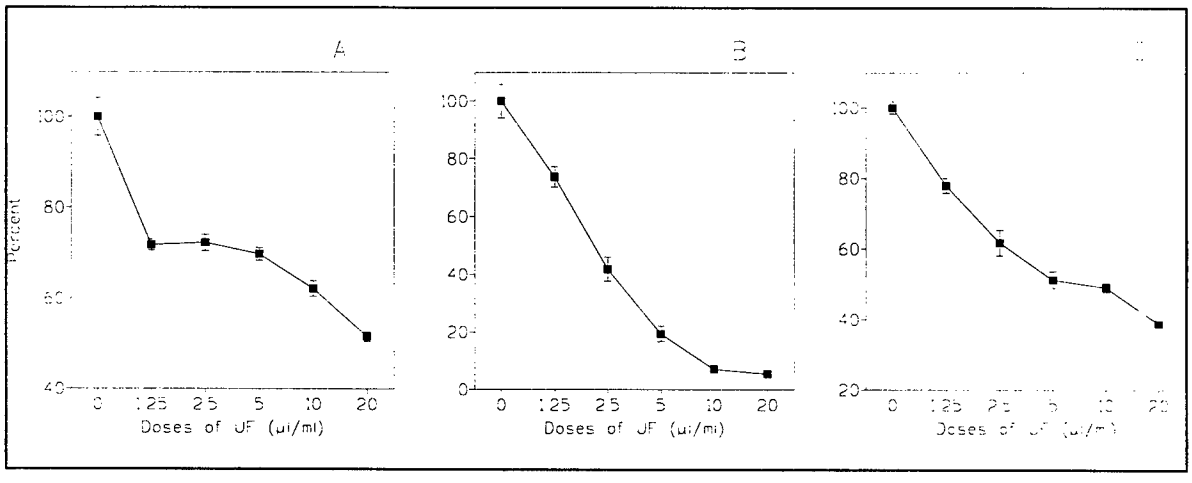

Figure 2. Effect of uterine fluid (UF) on MCF-7 (A) and Hs578T (B) and MDA 231 (C) cell DNA synthesis. Cells were grown as described in figure 1. Cells were treated with the indicated doses of UF (μl/ml) in serum free media for 24h. At the end of incubation, [^{3}H]-thymidine incorporation was determined as described (28). The experiments were repeated 3 times with similar results and the means of triplicate determinations were plotted as percent of control.

The addition of 2% UF into the culture resulted in a 50%, 95% and 61% inhibition of [^{3}H]-thymidine incorporation into MCF-7 (Fig. 2A), Hs578T (Fig. 2B) and MDA 231 (Fig. 2C) cell lines, respectively. The results from this study suggest that rat UF contains a very potent growth inhibitor for breast epithelial cells. Since the inhibitory activity was observed in both ER-positive and ER-negative cell lines, the inhibitor does not require the ER for its action.

The data are consistent with the hypothesis that UF contains a mammary growth inhibitor named uterine derived growth inhibitor (UDGI). The present work was conducted to characterize, identify, and purify the protein(s) primarily responsible for the described UDGI activity and to characterize its biological activity *in vitro*.

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Initial studies were conducted to determine whether a putative protein could be assigned to the peak (s) of UDGI biological activity in order to assess the feasibility of purification and to focus on a particular protein for preparative purification. Accordingly, gel filtration chromatography and reverse phase HPLC were used to analyze UDGI activity, and the eluted proteins were analysed by SDS-PAGE. UDGI activity was collected from rat UF following estradiol implantation, as described in "Experimental Procedures".

Ion exchange chromatography was initially performed to analyse the biological activity of UDGI. The samples were dialysed against 20 mM of ammonium carbonate (pH 8.85), applied to and eluted from a DE-52 anion-exchange column according to the methods described in "Experimental Procedures". Fig. 3 shows the biological activity and A_{280} protein elution profiles. The elution of the major growth inhibitory peak was observed from estradiol-induced rat UF.

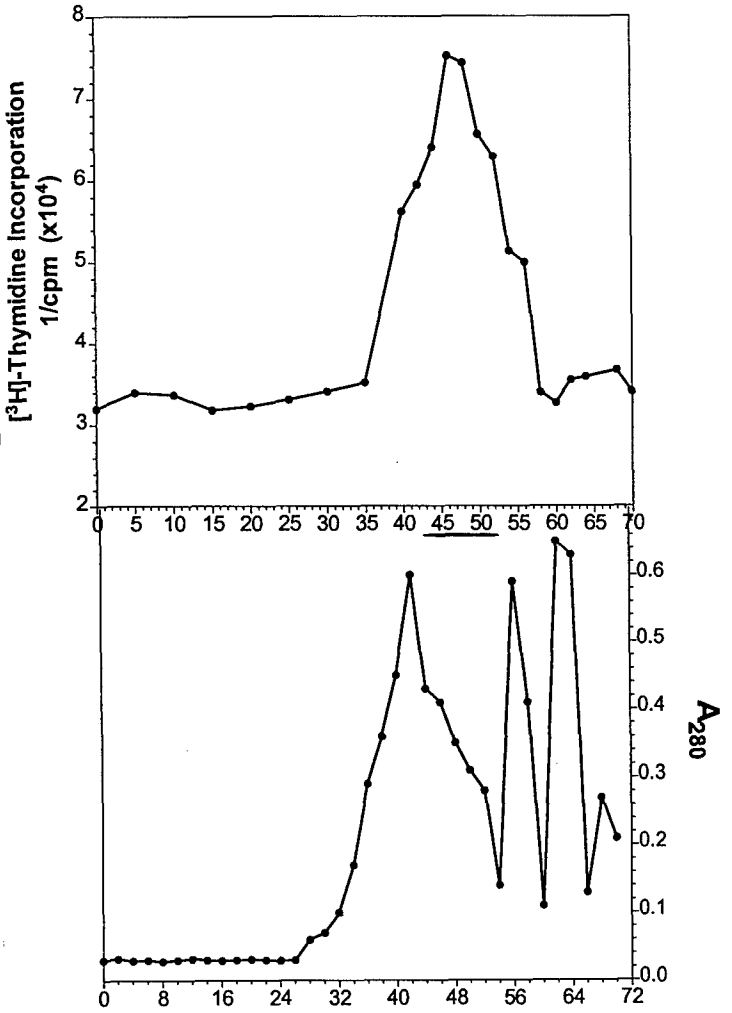

FIGURE 3. **Ion exchange chromatography**. Proteins from rat UF were chromatographed through a DE-52 anion exchange column. 100 μl aliquots from each fraction were vacuum-dried and assayed for inhibition of [^{3}H]-thymidine incorporation in Hs578T cells as described in "Experimental Procedures". Activity was plotted as the reciprocal of incorporated counts/min (1/cpm) to illustrate the inhibition of [^{3}H]-thymidine incorporation as a peak of activity. Activity eluted as a single peak and the fractions were pooled and processed for analytical gel filtration chromatography and HPLC, as described in other figures. The bottom panel shows the corresponding A_{280} pattern of the total protein.

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Fractions from the ion exchange chromatography representing the major peak of the eluted biological activity (Fig. 3, 42 to 52) were collected and pooled. The pooled sample was dialysed against 1 M of acetic acid (pH 2.5) overnight at 4^0C. The dialysed samples were quickly frozen, lyophilized, and stored at -20 ^{0}C. UDGI activity prepared in this manner was further analysed by gel filtration chromatography for assignment of size using a variety of buffers including acetic acid, ammonium carbonate, and ammonium acetate. Of these buffers, gel filtration chromatography in 1 M of acetic acid optimally reduced any interaction with the column matrix and allowed for a reproducible recovery of an activity peak as shown in Fig. 4. The biological activity was detected as a single peak, eluting consistently in the calculated 21-22 kDa size range. SDS-PAGE analysis of the eluted fractions (Fig. 4, lower panel) showed the elution pattern of a 21 to 22 kDa protein to be directly correlated to the elution peak of biological activity. To further establish the correlation of this protein species with peak activity, additional samples were pooled, chromatographed through C18 reverse phase HPLC columns, and eluted with a linear gradient of acetonitrile as shown in Fig. 5. In direct agreement with gel filtration, the major peak of UDGI from HPLC was associated with a protein of approximately 21-22 kDa, as analysed by SDS-PAGE (Fig. 5, lower panel, arrow, fractions 80 to 86).

Owing to the ability of HPLC to separate proteins of similar size based on hydrophobic properties, the last purification step utilized reverse phase HPLC. Pooled fractions from gel filtration chromatography were vacuum-dried, resolubilized in 50% formic acid, and analysed using reverse phase HPLC, as described in "Experimental Procedures". The column was eluted with a shallow gradient of acetonitrile to produce optimal separation of the major peak *versus* minor peak proteins. Fig. 6 shows the biological activity elution profiles and the corresponding SDS-PAGE analysis with peak activity. The biological activity eluted as a well defined peak which in turn, was directly correlated with the elution pattern of the 21-22 kDa protein species (Fig. 6, lower panel). This protein was purified to near homogeneity, as determined by SDS-PAGE analysis and silver staining. The purification procedure as described yielded approximately 500-700 ng of the 21-22 kDa protein from 2 ml of UF.

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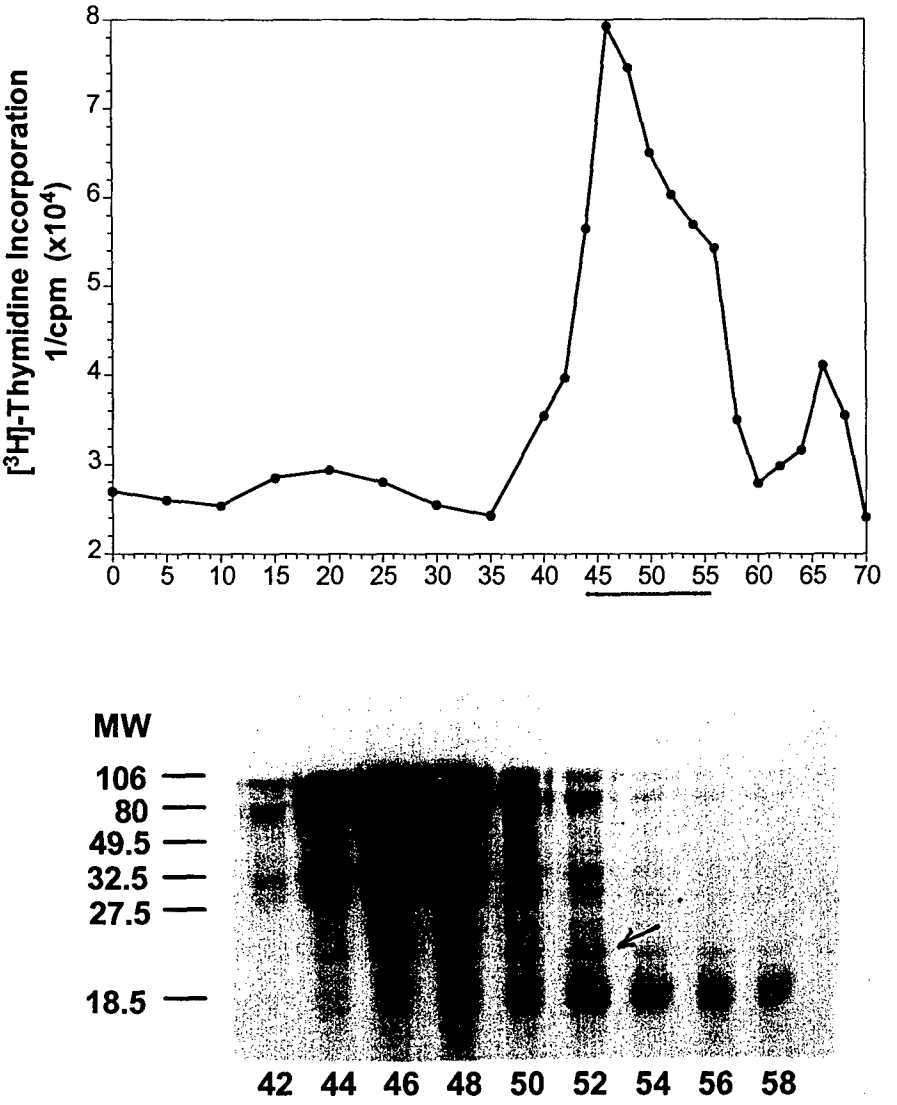

FIGURE 4. **Analytical gel filtration chromatography**. Proteins from the UF were chromatographed through a DE-52 anion exchange column, as described in Fig. 3, and analysed by gel filtration chromatography. The fractions were assayed for activity with Hs578T cells, as described in Fig. 3 and in "Experimental Procedures". Upper panel, elution profile of the biological activity from the P-100 gel filtration column. The biological activity eluted as a single peak (maximum activity at fractions 46 to 52) is associated with the 21-22 kDa size region. Lower panel, SDS analysis of eluted fractions. Fractions (42 to 58) from gel filtration were vacuum-dried, electrophoresed through a 16% acrylamide gel, and stained using the silver method. The elution pattern of a 21-22 kDa species (lower panel, arrow, fractions 46 to 52) correlated directly with the position of the eluted bioactivity peak. Molecular size markers are shown.

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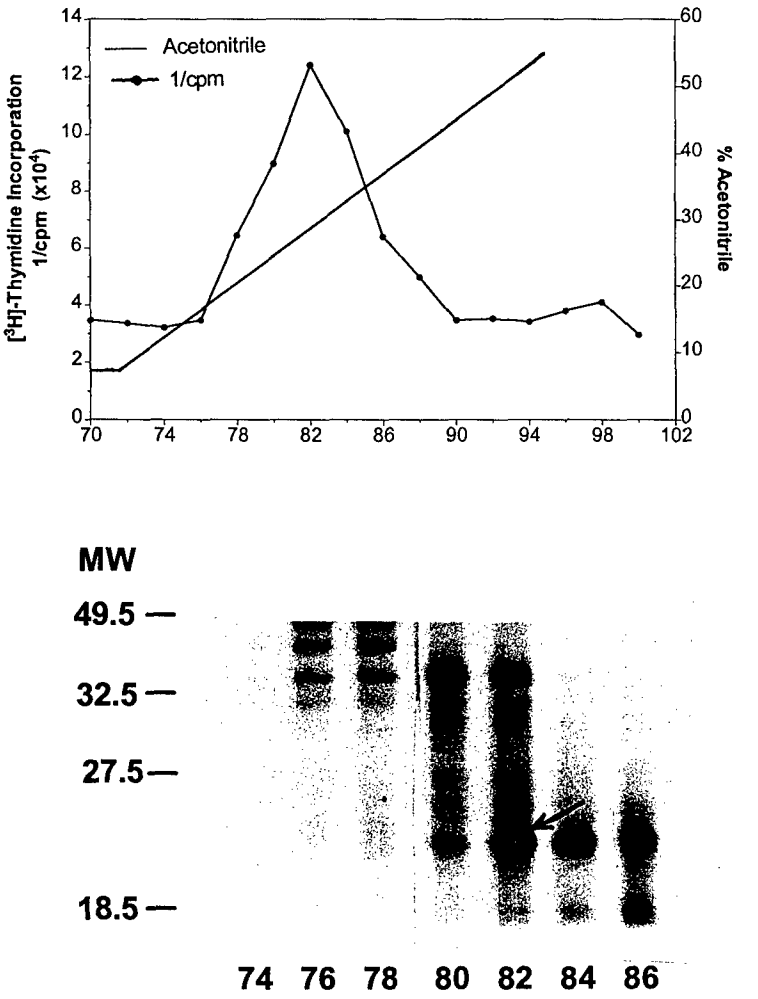

FIGURE 5. **Analytical reverse phase HPLC**. Upper panel, proteins were chromatographed with a C-18 reverse phase column, as described in Fig. 3 and in "Experimental Procedures". 100 μl aliquots from each fraction were vacuum-dried and assayed for inhibition of [^{3}H]-thymidine incorporation in Hs578T cells, as described in "Experimental Procedures". The biological activity eluted as a single peak (maximum activity at fractions 80 to 86) is associated with the 21-22 kDa size region. Lower panel, SDS analysis of eluted fractions. Fractions (74 to 86) from gel filtration were vacuum-dried, electrophoresed through a 16% acrylamide gel, and stained using the silver method. The elution pattern of a 21-22 kDa species (lower panel, arrow, fractions 80 to 86) correlated directly with the position of the eluted bioactivity peak. Molecular size markers are shown.

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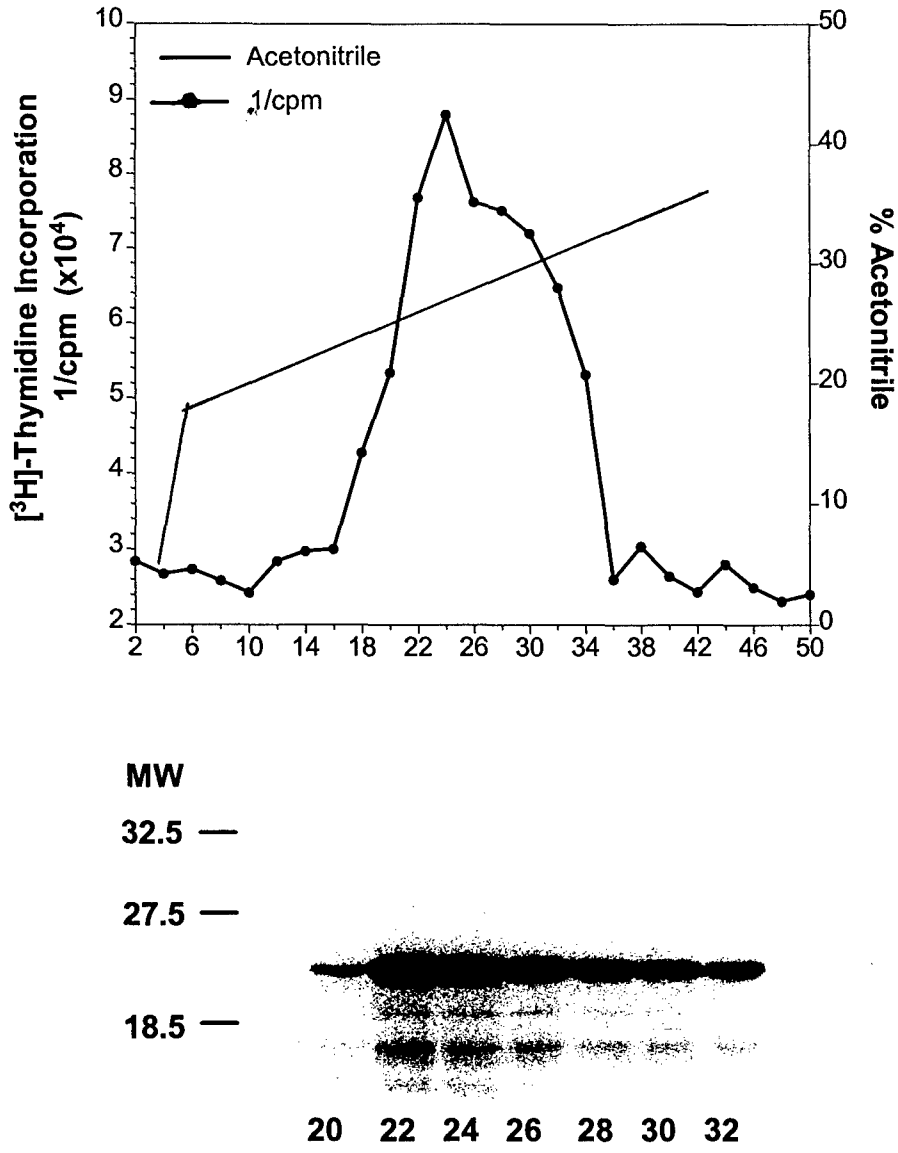

FIGURE 6. **Preparative HPLC chromatography**. Upper panel, fractions from gel filtration chromatography were pooled and used for reverse phase HPLC as described in "Experimental Procedures". Shown is the elution profile of the biological activity with Hs578T cells and an acetonitrile elution gradient. The biological activity eluted with a consistently observed peak (fractions 20 to 32). The lower panel shows the SDS-PAGE analysis and silver staining of the proteins. The elution pattern of the 21-22 kDa protein correlated with the initial peak (fractions 20 to 32) and was purified to near homogeneity. Molecular size markers are shown.

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For microsequence analysis, purified UDGI protein was electrophoresed through SDS-PAGE gels, blotted to PVDF membranes, and analysed for amino-terminal sequence. A single sequence was detected with unambiguous assignments made for the position 1-23. Analysis with SwissProt, SPUpdate, GenPept, and GPUpdata data bases indicated that this region was 96% homoloous with a previously characterized protein termed urogenital sinus derived growth inhibitor sp20 (29). The difference in homology between the sp20 protein and the protein identified in this study owes to one amino-acid at position 16 (as illustrated below)..

UDGI: NH-2-Thr-Trp-Glu-Ala-Met-Leu-Pro-Val-Arg-Leu-Ala-Glu-Lys-Ser-Gln-Val-Glu-Glu-Val-Ala-Ala-Thr-Gly

ps20: NH-2-Thr-Trp-Glu-Ala-Met-Leu-Pro-Val-Arg-Leu-Ala-Glu-Lys-Ser-Gln-Arg-Glu-Glu-Val-Ala-Ala-Thr-Gly

To confirm whether UDGI and ps20 were derived from the same cDNA, we degenerate a UDGI primer corresponding to amino acids 10 to 17 (which is 3 nucleotides different from the published sequence for ps20) and used a PCR strategy in combination with vector primer to amplify a fragment of UDGI cDNA from the rat pcDNA3.1 uterus cDNA library. A 800 bp fragment was produced by PCR. This fragment was further cloned into the pcDNA3.1 vector and sequenced. The deduced amino acid sequence of this clone matched directly with the first 23 amino acid sequence of UDGI and shared 98% identity to the amino acid sequence of ps20 protein (29). The results suggest that UDGI is likely the ps20 protein.

To examine UDGI gene expression *in vivo*, Northern blot analysis was performed on total RNA isolated from rat mammary tissue at different stage of pregnancy. Despite previous reports of a single transcript for ps20 gene (29), two transcripts of aproximately 1.4 and 1.1 kb were clearly detected by the UDGI cDNA probe (Fig. 7). This observation raised the questions of whether the 1.4 and 1.1 kb transcripts were transcribed from a single gene or a different gene, and whether the difference in transcript size observed was due to differential splicing or difference in the 5' or 3' untranslated sequences.

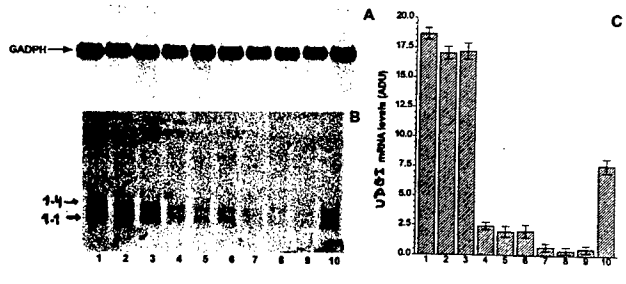

Figure 7. Expression of UDGI in the mammary gland during different stages of pregnancy, during lactation and involution. Mammary tissues were non-pregnant (lane **1**), 3 days (lane **2**), 6 days (lane **3**), 9 days (lane **4**), 12 days (lane **5**), 15 days (lane **6**) and 18 days (lane **7**) of pregnancy; 2 days (lane **8**) and 5 days (lane **9**) of lactation; and 2 days after pups were removed (lane **10**). Total RNA derived from mammary gland was subjected to Northern blot. Blots were hybridized with GAPDH (**A**) and rat UDGI (**B**) cDNAs. Densitometric scanning of the UDGI bands are shown in (**C**). Note that two transcripts for UDGI were detected.

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When Northern analysis was performed on total RNA isolated from various rat tissues, two UDGI transcripts were also detected by UDGI cDNA (Fig. 8). The ratio of these transcripts varied from tissue to tissue. Of multiple tissues survey, the highest levels of UDGI mRNA was observed in the lung (Fig. 8). The 1.4 kb transcript was the predominant species of UDGI transcript in bladder and red muscle.

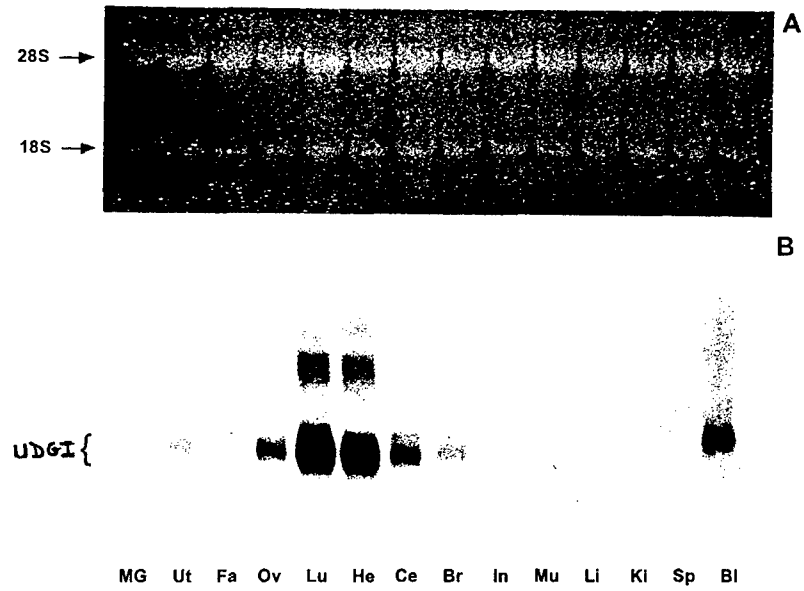

Figure 8. Northern blot analysis of UDGI gene expression in female adult rat tissues. Total RNA derived from various tissues of 3 month old female rat was subjected to Northern blot. Blots were hybridized with and GAPDH (**A**) and rat UDGI (**B**) cDNAs.

To extend our study regarding the function of UDGI expression (ps20) in growth regulation of normal mammary cells and breast cancer cells, we developed a rabbit polyclonal antibody against purified UDGI protein. When uterine fluid (the fluid from which UDGI was identified) was analysed by Western analysis using UDGI antibody, three specific bands of apparent molecular mass 24, 27 and 29 kDa were specifically recognized by UDGI antibody (Fig. 9). The 27 kDa species was the major species. Preabsorbed control gave no signal. Similar results were observed when conditioned media from primary mammary cells was analysed by Western analysis. However, the 24 kDa form became the major species when cell extracts were used. The results suggest that UDGI may undergo post-translational modification.

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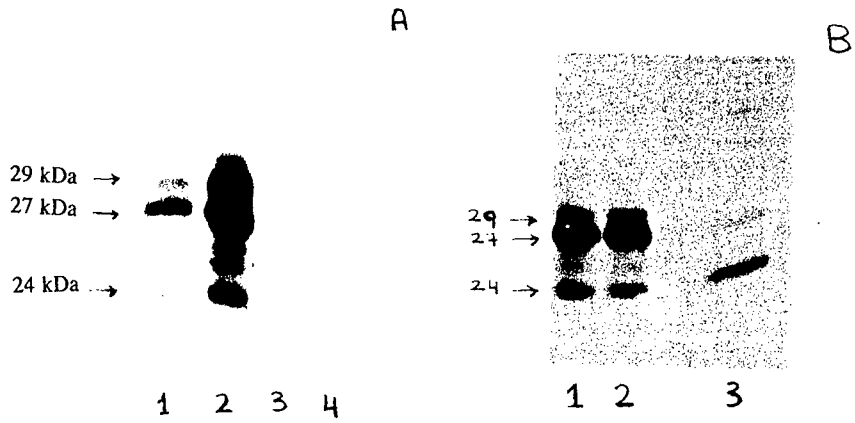

Figure 9. Western blot analysis of purified UDGI, rat primary cell conditioned media and rat primary cell extract. Proteins derived from uterine fluid (lane 1A), purified UDGI (lanes 2A-4A), rat primary conditioned media (Lanes 1B and 2B) and rat primary cell extract (Lane 3B) were subjected to Western analysis. Blots were incubated with either anti-UDGI antibody (Lanes 1A, 2A, 1B-3B), or preabsorbed UDGI antibody (lane 3A), or preimmune serum (lane 4A). Molecular weight of immuno-reactive bands are shown.

To determine if post-translational modification plays a role in UDGI activity, the UDGI open reading frame (without the signal peptide) was subcloned into a bacterial expression vector and expressed as a carboxyl-terminal histidine-tagged protein in bacteria (Fig. 10). Western analysis with UDGI antibody demonstrated specific immunoreactive with recombinant UDGI protein (data not shown).

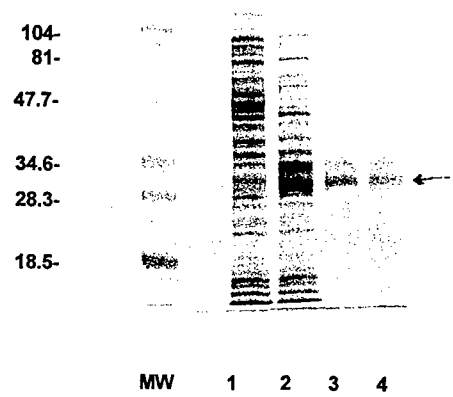

Figure 10. Expression of UDGI-His-tagged protein. Crude extracts of bacterial cells prior to induction (lane 1), following induction (lane 2), and after purification (lanes 3 and 4) were resolved by SDS-PAGE and visualized by Coomassie Blue stain.

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Because Hs578T and MCF-7 cells are target cell types shown to be sensitive to purified UDGI, MCF-7 and Hs578T cells were assayed for UDGI-induced inhibition of [^{3}H]-thymidine incorporation. As shown in Figure 11, recombinant UDGI induced only a minor inhibition of [^{3}H]- thymidine incorporation in both MCF-7 and Hs578T cells. The data suggest that post-translational modification of UDGI may be required for full activity.

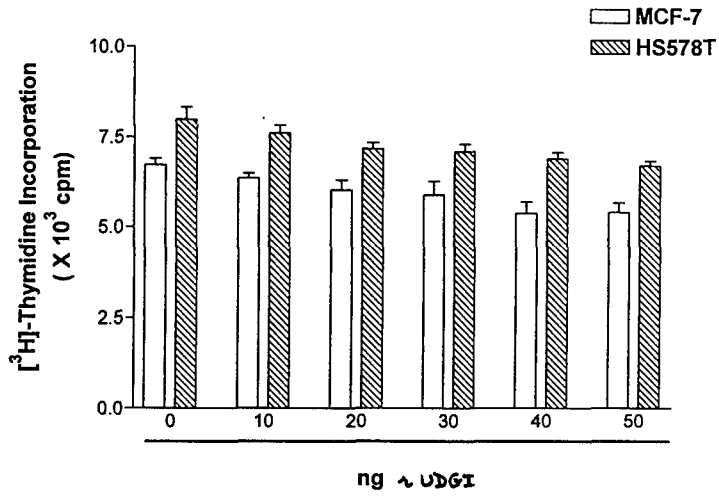

Figure 11. Effect of recombinant UDGI on MCF-7 and Hs578T cell DNA synthesis. Cells were grown as described in Figure 1. Cells were treated with the indicated doses of recombinant rat UDGI (ng/ml) in serum free media for 24h. At the end of incubation, [^{3}H]-thymidine incorporation was determined as described (28). The experiments were repeated 3 times with similar results and the means of triplicate determinations were plotted as percent of control.

Since growth of mammary epithelial cells in the mammary gland is influenced, to some extent by female steroid hormones, and purified UDGI inhibits breast cancer cells *in vitro*, Northern and Western blot analyses were used to determine the expression of UDGI gene in the mammary gland of 2 and 14 month old female rats. In addition, the expression of UDGI gene in normal mammary tissue and tumours, prior to and after antiestrogen treatment, was also investigated. The UDGI gene expression was then correlated with proliferation, apoptosis or tumour volume. Given the extensive use and efficacy of antiestrogens in breast cancer treatment, and ongoing research regarding their usefulness in breast cancer prevention and treatment, new data regarding the action of ICI and tamoxifen on UDGI expression deserve investigation.

In 2 month old rats, estradiol caused mammary epithelial cell proliferation (Fig. 12) concomitant with suppression of UDGI expression (Fig. 13 and 14). Pure anti-estrogen ICI 182780, which blocked breast epithelial cell proliferation and caused apoptosis (Fig. 12), greatly stimulated UDGI expression (Fig. 15 and 14). Mild stimulation of UDGI expression by tamoxifen was also observed (Fig. 16 and 14). The observations indicate that, *in vivo,* UDGI expression is associated with growth inhibition and apoptosis.

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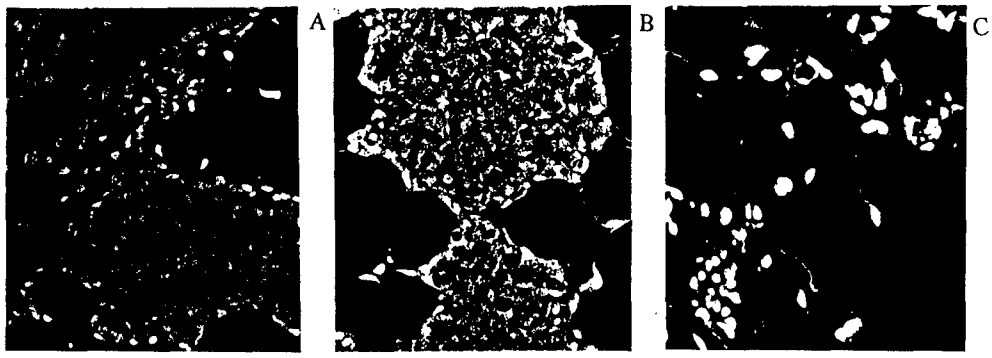

Figure 12. Effects of ICI and estradiol on mammary epithelial cell apoptosis. Rats were treated with either vehicle or estradiol or ICI 182780. Five μM sections of mammary gland were used. Fragmented DNA were labelled using the ApoAlert DNA fragmentation assay (Clontech Laboratories, Palo Alto, CA). Control mammary sections (**A**), estradiol-treated mammary sections (**B**) and ICI-treated mammary sections (**C**).

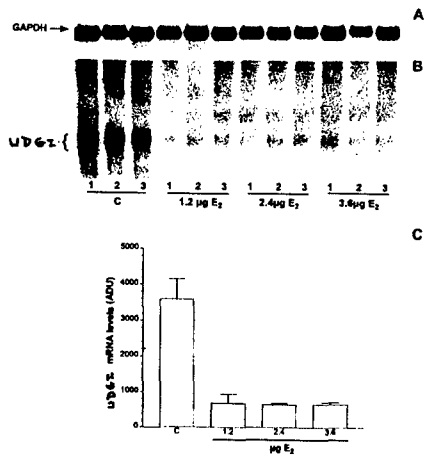

Figure 13. Effects of estradiol on mammary gland UDGI expression. Rats were treated with indicated concentrations of 17-ß estradiol. Total RNA derived from mammary glands was subjected to Northern blot. Blots were hybridized with and GAPDH (**A**) rat UDGI (**B**) cDNAs. Densitometric scanning of the UDGI band is shown in (**C**). Note that UDGI expression was severely inhibited by estradiol treatment.

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Figure 14. Effects of tamoxifen, ICI 182780 and estradiol on mammary gland UDGI expression. Rats were treated with indicated concentrations of tamoxifen (**TAM**), ICI 182780 (**ICI**), and 17-ß estradiol (E_2) for 3 weeks. Total proteins derived from mammary glands was subjected to Western blot analysis. Blots were blotted with tubulin (**A**) and rat UDGI (**B**) antibodies. Note that tamoxifen and ICI significantly induced UDGI expression.

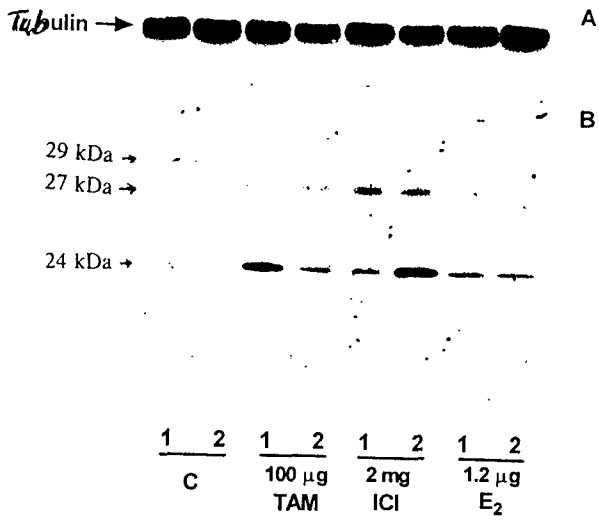
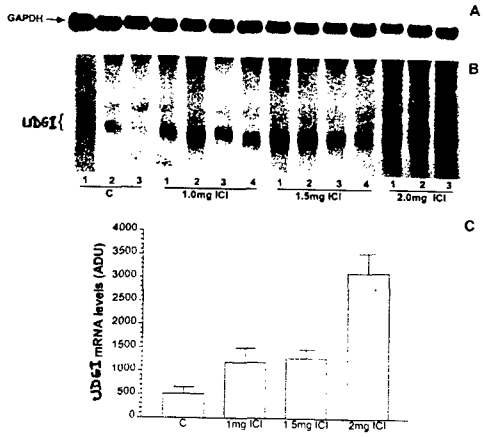


Figure 15. Effects of ICI 182780 on mammary gland UDGI gene expression. Rats were treated with indicated concentrations of ICI. Total RNA derived from mammary glands was subjected to Northern blot. Blots were hybridized with GAPDH (**A**) and rat UDGI (**B**) cDNAs. Densitometric scanning of the UDGI bands are shown in (**C**).



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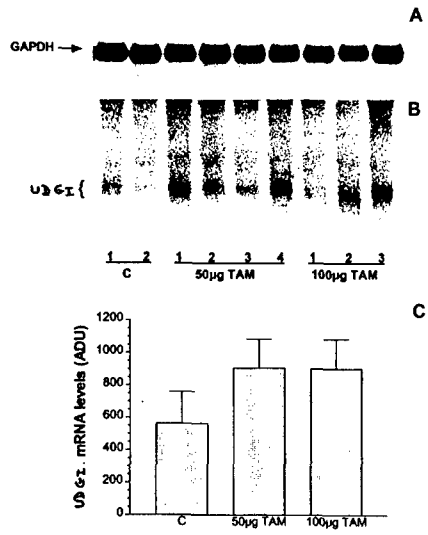

Figure 16. Effects of tamoxifen on mammary gland UDGI expression. Rats were treated with indicated concentrations of tamoxifen. Total RNA derived from mammary glands was subjected to Northern blot. Blots were hybridized with and GAPDH (**A**) rat UDGI (**B**) cDNAs. Densitometric scanning of the UDGI band is shown in (**C**).

Surprisingly, UDGI mRNA was barely detectable in mammary glands of 14 month old rats (Fig. 17). The loss of UDGI gene expression was completely restored by treament with antiestrogen ICI 182780. Since UDGI is a potent growth inhibitor for breast epithelial cells *in vitro*, the decrease in UDGI expression in mammary gland of old animals may allow the tumour cells to grow. This hypothesis is supported by the fact that high breast cancer incidence is seen in old animals and post-menopausal women.

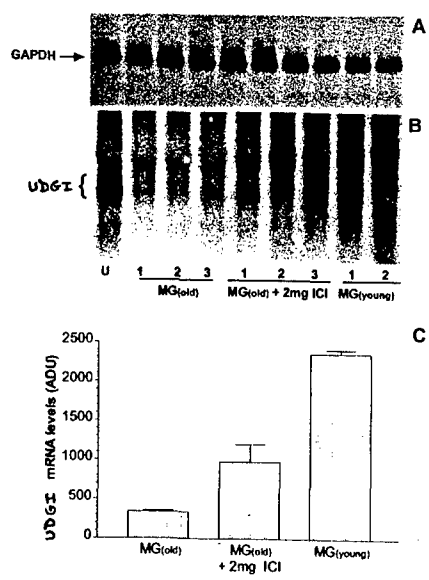

Figure 17. Stimulation of UDGI expression in mammary tissue of old rats. Fourteen-month old rats were treated with indicated concentrations of ICI 182780. Total RNA derived from mammary glands was subjected to Northern blot. Blots were hybridized with and GAPDH (**A**) rat UDGI (**B**) cDNAs. Densitometric scanning of the UDGI band is shown in (**C**). Samples are positive control uterus RNA (**U**), mammary RNA of 2 month old rat (**MG (young)**), and mammary RNA of 14 month old rats (**MG (old)**). Note that the UDGI transcripts were barely detectable in mammary glands of old rats. A significant increase in UDGI gene expression was seen following ICI administration.

Assuming that UDGI is a growth inhibitor and apoptotic factor for breast cells, we predicted that UDGI expression in breast tumours would be low. In addition, we wanted to determine whether we could reactivate or induce UDGI expression in mammary tumours by any anti-tumour agents. Agents that can reactivate UDGI expression would be considered for breast cancer treatment or prevention. It is well known that a significant proportion of DMBA-induced rat mammary tumours are antiestrogen-responsive (26,30). Our first attempt was to use the DMBA mammary tumour model (26) to determine if there was a correlation between the antineoplastic activity of ICI 182780 and tamoxifen with the ability of these agents to induce UDGI gene expression. As predicted, UDGI transcripts were barely detectable in all DMBA tumours examined (Fig. 18). UDGI gene expression was greatly stimulated following either tamoxifen or ICI 182780 treatment for 2 weeks (Fig. 18B). The induction was positively correlated with the ability of these agents to suppress tumour growth, as determined by tumour volume (Fig. 18D).

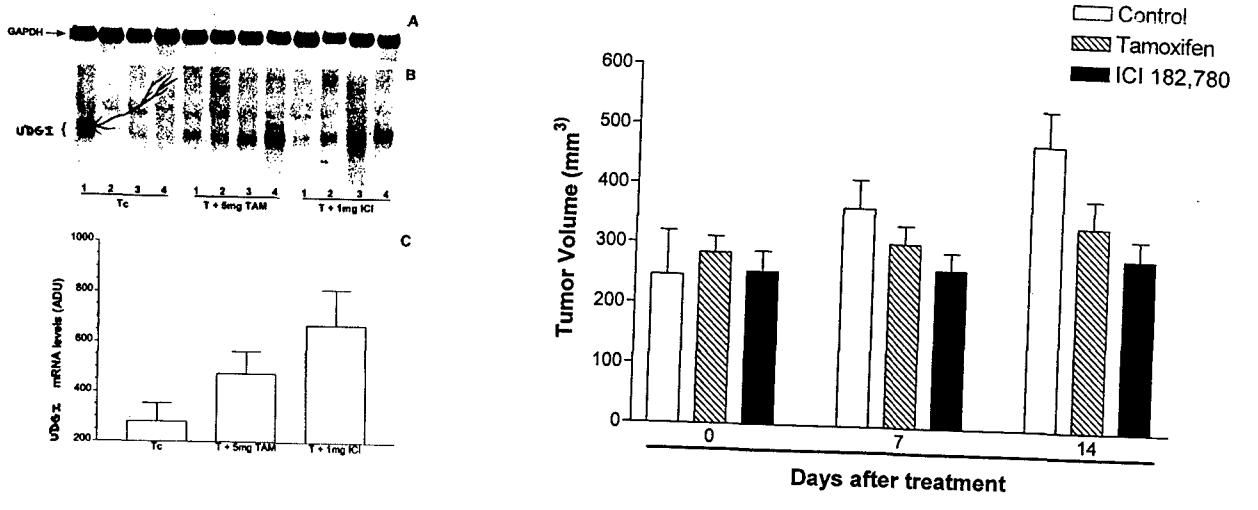

Figure 18. Reactivation of UDGI expression in rat DMBA mammary tumours. DMBA mammary tumours were created as previously described (26). Rats bearing mammary tumours were either left untreated (**Tc**) or treated with indicated dose of tamoxifen (**TAM**) or indicated doses of ICI 182780 (**ICI**) for two weeks. After two weeks, tumours were collected and total RNA was extracted and subjected to Northern blot. Blots were hybridized with and GAPDH (**A**) rat UDGI (**B**) cDNAs. Densitometric scanning of the UDGI band is shown in (**C**).Tumour volume was measured weekly and plotted as shown in (**D**). Note that the UDGI transcripts were barely detectable in DMBA mammary tumours. Significant increase in UDGI transcripts were observed following either tamoxifen or ICI treatment.

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To further demonstrate the role of UDGI protein in regulating mammary cell growth, human breast cancer MCF-7 cells were transfected with a mammalian expression vector containing full length UDGI cDNA (UDGI-pcDNA3.1) or with control pcDNA3.1 vector. Conditioned media (CM) from stable transfectant lines was collected, concentrated and analysed by Western blot analysis with UDGI antibody. As predicted, the 24, 27 and 29 kDa proteins were detected in the CM of MCF-7 cells transfected with UDGI cDNA, but not in CM of mock-transfected MCF-7 cells (Fig. 19). When these cells were grown in medium containing 10% FCS, there was no noticable difference in growth rate between the UDGI transfectant cell lines and the mock transfectants. When the UDGI transfectants were deprived of serum, they exhibited an extensive array of filopodia and rounded up (Fig. 20). By 48h after serum starvation, extensive cell death was observed in UDGI transfectant lines as compared to mock transfectant lines (Fig. 20).

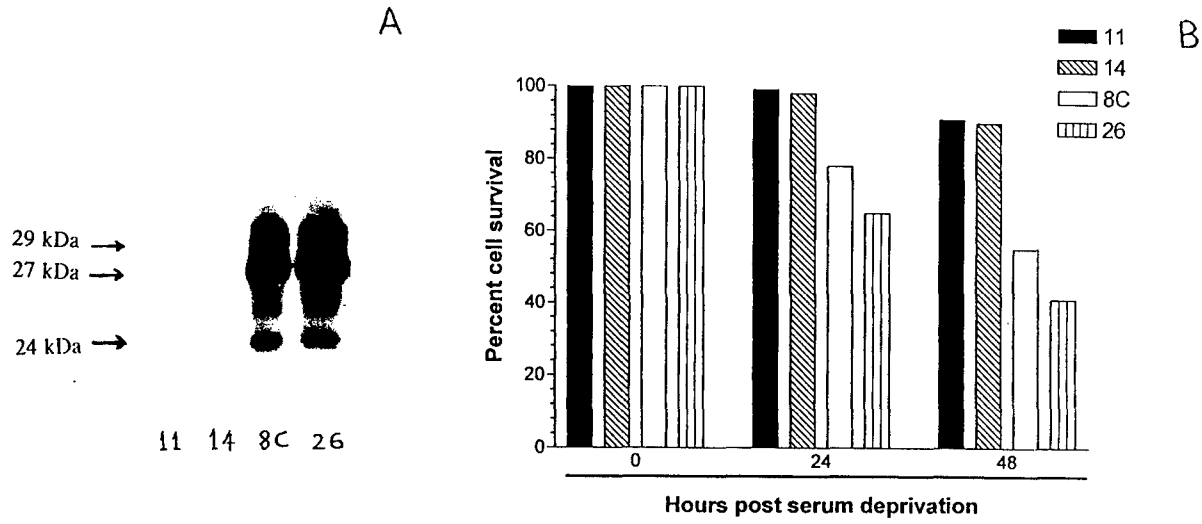

Figure 19. Overexpression of UDGI accelerates cell death. (**A**) Western blot analysis of UDGI secretion into conditioned media of mock- transfected lines (11 and 14) and two clones expressed UDGI (8C and 26) using UDGI antibody. (**B**) Viability assays. Cultures of pcDNA3.1 mock-transfected lines (11 and 14) and two clones expressed UDGI (8C and 26) were deprived of serum. The viability was assessed by trypan blue exclusion at 24 and 48 hours following serum deprivation and was plotted as percent cell survival.

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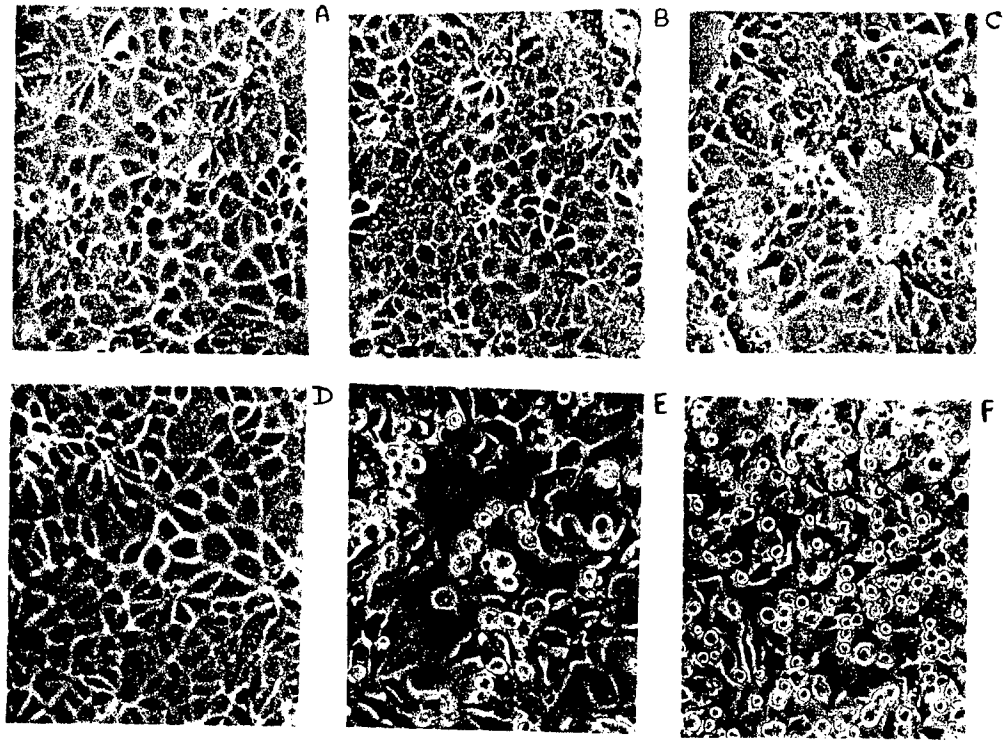

Figure 20. Morphology of UDGI transfectant cells before and after serum starvation. Representative photographs of mock transfected cell line 11 (**A, B, C**) and UDGI-transfected cell lines (**D, E, F**) at 0 (**A and D**), 24h (**B and D**) and 48h (**C and F**) following serum deprivation. X250.

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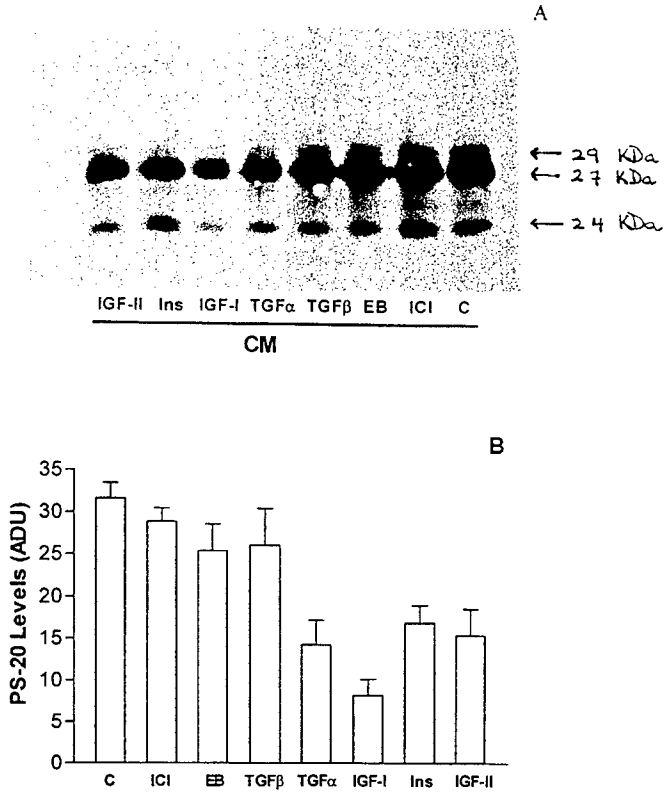

Figure 21. Effects of IGFs, insulin, TGF-ß, TGF-α, ICI 182780 and vitamin D analog EB1089 on UDGI accumulation in conditioned media of primary rat mammary gland cells. Primary mammary cells were isolated as described previously (19). Cells were treated with 20 ng/ml IGF-II, 5 μg/ml insulin (Ins), 20 ng/ml IGF-I, 10 ng/ml TGF-α, 10 ng/ml TGF-ß, 1 x 10^{-8} M EB1089 (EB) and 1 x 10^{-8}M ICI 182780 (ICI) for 48 hours. One ml of conditioned media was concentrated and was subjected to Western blot analysis. Membranes were blotted with rat UDGI antibody (**A**). Densitometric scanning of the UDGI bands is shown in (**B**). Note that TGF-α, IGF-I, IGF-II and insulin significantly reduced UDGI accumulation.

STATEMENT OF WORK

To date we have completed Task 1 to 5 of the Statement of Work outlined in the proposal: Purification of UDGI from rat uterine fluid, polyclonal antibody production, UDGI cDNA isolation, transfection of UDGI expression into breast cancer cells, regulation of UDGI gene in normal breast cells. In doing so we came across some unanticipated problems:

1. To analyse UDGI bioactive proteins, all chromatography buffers had to be both volatile (no salt residues upon drying) and bacteriostatic (sterile, non-supportive of bacterial growth) so that aliquots from gel filtration or HPLC columns could be vacuum-dried in sterile vials and used directly for biological assays (in addition to targeting breast cancer cells in culture) without additional steps of dialysis and sterilization of the sample. During the course of purification we faced several problems concerning the choice of eluting buffers that would meet our requirements.

2. A second problem arose in the reproducible recovery of an activity peak during purification. This was due to an interaction between the protein and the column matrix. To optimize it we had to select several buffers that would optimally reduce the interaction.

Presently we are in the process of and will have answers shortly on:

1. cDNA library screening to determine the difference between 1.1 and 1.4 kb transcripts. The human UDGI is also cloned.

2. *In vivo* tumorigenic assay.

3. Immunolocalization of UDGI in normal and mammary tumours.

4. Examine the the mechanism(s) of UDGI-induced apoptosis *in vitro*.

Tasks remaining:

Task 6. Detection of UDGI binding sites. Months 21-30:

a. Direct binding assays
b. Affinity labelling of UDGI receptors

CONCLUSION

In this annual report, we report the purification of UDGI, a uterine derived secreted protein which possesses growth inhibitory properties. Sequence analysis revealed that UDGI shares 98% identity to ps20 growth inhibitor protein which was previously isolated from urogenital sinus mesenchymal cells (29). UDGI (ps20) belongs to a family composed primarily of secreted serine protease inhibitors. Although purified UDGI was shown to potently inhibits breast cancer cell growth, recombinant UDGI only exhibited mild inhibitory effects. Polyclonal antibodies against purified UDGI recognized native UDGI from uterine fluid, conditioned media of primary mammary cells and stable UDGI-transfectant MCF-7 cell lines with molecular mass of 24, 27 and 29 kDa, respectively. A full length of UDGI cDNA was isolated from the rat uterus cDNA library. Northern blot analysis using UDGI cDNA detected two UDGI transcripts in rat mammary tissue using UDGI cDNA. UDGI mRNA levels gradually decreased as pregnancy advanced. UDGI mRNA was also detected in various female rat tissues with the highest expression in the lung and heart. *In vitro,* using primary mammary cells, UDGI expression was negatively regulated by IGF-I, IGF-II, insulin and TGF-α. *In vivo*, UDGI expression was greatly suppressed by estradiol while pure antiestrogen ICI 182780 and tamoxifen upregulated UDGI expression. UDGI gene expression was undectable in mammary tissue of 14 month old rats and its expression was greatly stimulated following ICI 182780 administration. The induction of UDGI correlated positively with apoptosis in mammary gland. In mammary DMBA tumours, UDGI gene expression was very low and was significantly induced following either tamoxifen or ICI 182780 treatment. Acceleration of apoptosis was observed in stable UDGI-transfectant MCF-7 cell lines following serum starvation. Taken together, these results indicate that UDGI is a growth inhibitor and apoptotic factor for breast epithelial cells, *in vitro*. *In vivo*, the low levels of UDGI expression in the mammary tissue of old rats and in breast tumours may facilitate tumour growth and progression. Induction of UDGI in normal mammary gland during apoptosis and in DMBA mammary tumours during tumour regression by antiestrogens suggest that UDGI may function as a mediator of local growth and apoptosis mechanisms.

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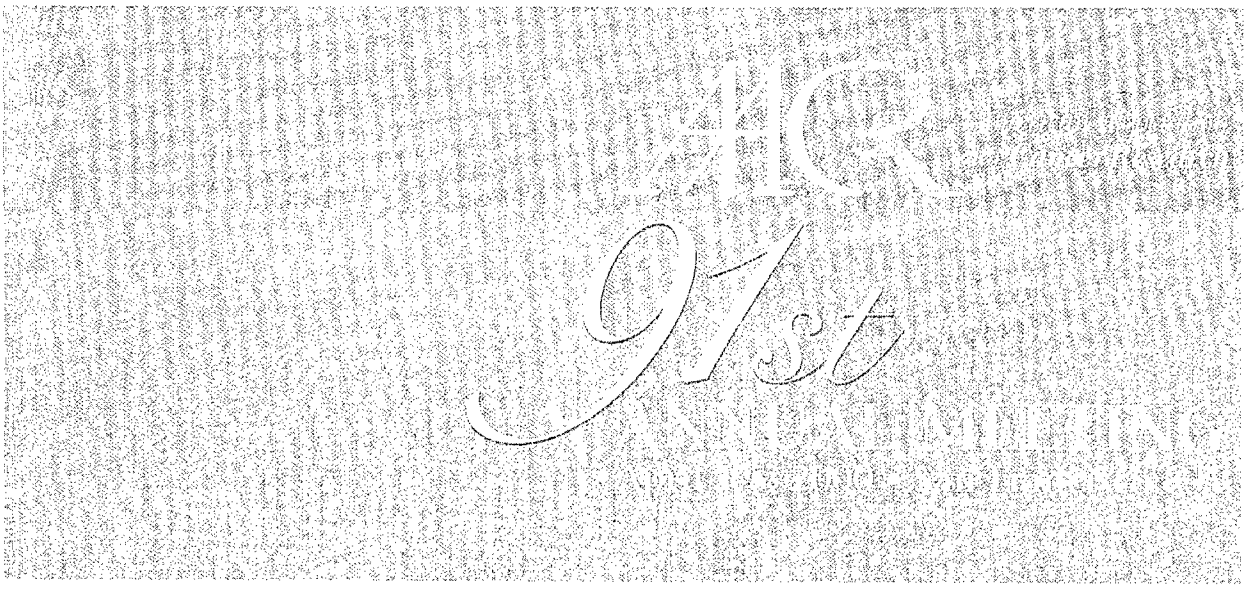
#5004 INHIBITION OF INSULIN-LIKE GROWTH FACTOR SIGNALING PATHWAYS IN MAMMARY GLAND BY PURE ANTI-ESTROGEN ICI 182780.

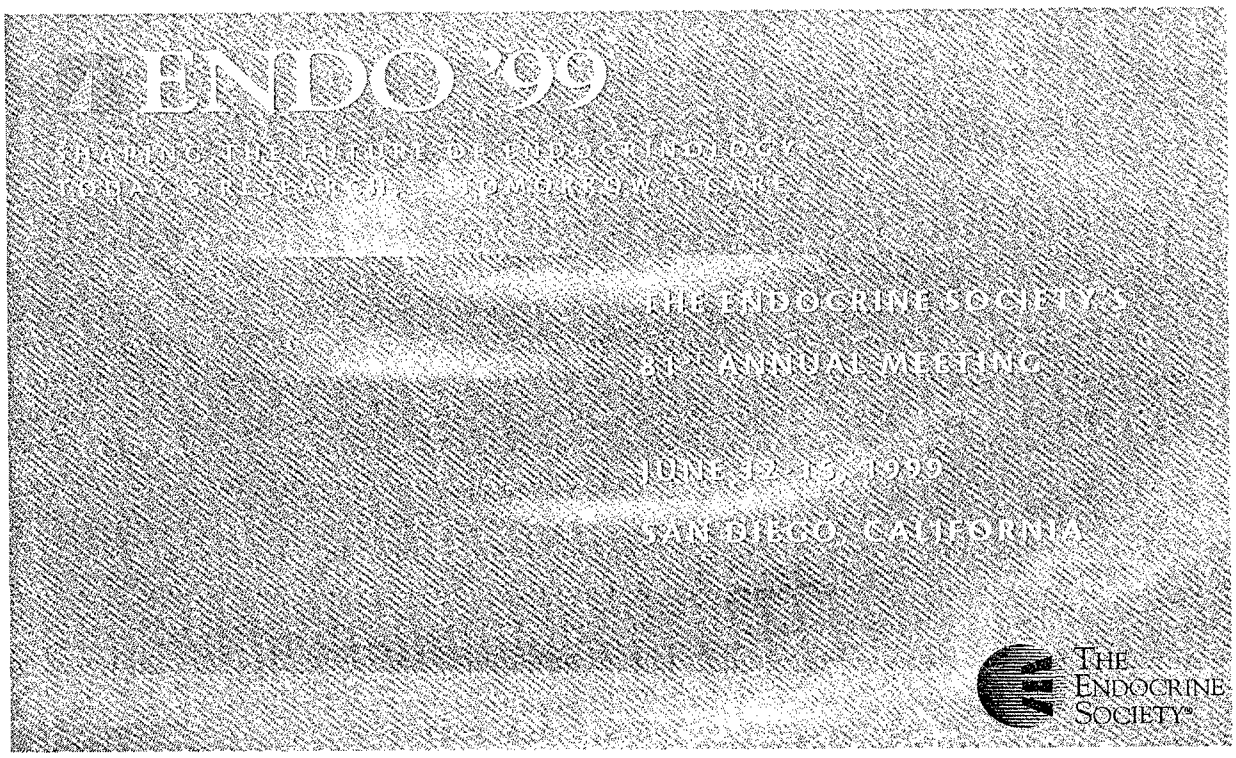
Hung T Huynh, *National Cancer Ctr, Singapore, Singapore*

The pure antiestrogen ICI 182780 (ICI) exhibits effects on some patients following disease progression on tamoxifen and proves to be clinically useful for the treatment of estrogen receptor positive breast tumours. We have assessed the *in vivo* effects of ICI on the IGF signaling pathway in the mammary gland of a rat model. Treatment of rats with 1, 1.5 and 2 mg ICI/kg BW/week resulted in a 2, 7 and 8 fold increase in insulin-like growth factor binding protein (IGFBP-3) transcripts. A slight decrease in IGFBP-4 gene expression was observed while IGFBP-2 and IGFBP-5 gene expression was significantly inhibited. ICI decreased IGF-IR and its basal phosphorylation to approximately 30% of the control mammary glands. IRS-1 and IRS-2 levels in the ICI-treated mammary glands were about 30% and 15% of controls, respectively. Phosphorylation of IRS-1 and IRS-2 proteins were barely detectable following ICI treatment. Despite significant reduction in phosphorylation of IGF-IR, IRS-1, IRS-2, the phospho p42/p44 MAPK levels were slightly decreased. Degradation of phospho p42/p44 MAPK were observed. PI-3 kinase p85 levels were not affected by ICI treatment while basal phospho PI-3K p85 was inhibited. Expression of Raf-1 protein was also inhibited by ICI treatment. Because the IGF system plays an important role in breast epithelial cell proliferation and apoptosis, the described activities of ICI may contribute to its anti-proliferative properties seen clinically and in animal models.



#2327 CLONING AND CHARACTERIZATION OF A NOVEL PREGNANCY-INDUCED GROWTH INHIBITOR. Hung T Huynh, *National Cancer Ctr, Singapore, Singapore*

Growth factors and growth inhibitors play an important role in the regulation of growth and differentiation of mammary epithelial cells. It is well known that during pregnancy, with the onset of terminal differentiation, the proliferation of mammary epithelial cells dramatically decreases. We report the cloning and characterization of a novel pregnancy-induced cDNA (namely OKL38)from a human ovarian cDNA library. This cDNA encodes a protein of approximately 38 kDa. Northern blot analyses from various tissues revealed the distribution of *OKL38* transcripts in most tissues, with the highest level in the ovary, kidney and liver. *OKL38* gene expression gradually increases as pregnancy advances and as induced by hCG, which is known to induce mammary gland differentiation. Expression in human breast cancer cell line was low and barely detectable in all breast tumour tissues examined. Growth inhibition induced by taxol and doxorubucin in breast cancer cells MCF-7 was associated with an increase in the *OKL38* gene expression. Transfection of *OKL38* cDNA into MCF-7 cells resulted in growth inhibition. The above observations suggest that *OKL38* may play a crucial role in the growth regulation and differentiation of the breast epithelial cells during pregnancy and tumorigenesis.



P1-277

ANDROGEN SENSITIVE GENE (ASG), A NOVEL GENE ASSOCIATED WITH CELL PROLIFERATION AND IS REGULATED BY STEROID HORMONES.

Daniela Marcantonio,* Hung Huynh. Department of Medicine, Lady Davis Institute for Medical Research, Montreal, QC, Canada

Using differential display, a novel gene approximately 3.8 Kb in length designated androgen sensitive gene (ASG) was cloned from a rat uterus cDNA library. The cDNA of ASG encodes for a protein of 385 amino acids and has a predicted size of 42 kDa. The predicted amino acid sequence of ASG contains potential nuclear targeting sequences suggesting that this protein may function in the nucleus. Northern blot analysis on various rat tissues indicated that ASG is expressed in cerebellum, pituitary gland, uterus, ovary, prostate, mammary gland, heart, bladder, spleen, fat and liver. Levels of ASG mRNA are highest in fat tissue. In rat mammary gland primary culture cells, ASG expression was positively regulated by IGF-I and IGF-II. Pure antiestrogen ICI 182780 positively regulated ASG gene expression in vivo and in vitro. In the rat prostate, ASG expression was upregulated by androgen depletion and under diabetic conditions. Subsequent androgen or insulin replacement respectively returned gene expression to control levels. In the rat uterus, ASG expression was inhibited in a dose dependant manner by estradiol whereas ICI 182780 increased gene expression of ASG. Our results show that ASG expression is regulated by steroid hormones and is inversely correlated with cell proliferation in vivo in the prostate, mammary gland and uterus. This suggests that ASG may play an important role in controlling cell growth by steroid hormones.

Appendix 5

DAVIES COLLISON CAVE
PATENT & TRADE MARK ATTORNEYS

25 May, 2000

Dr Hung Huynh
Principle Investigator
Laboratory of Molecular Endocrinology
Division of Cellular and Molecular Research
National Cancer Centre of Singapore
11 Hospital Drive
SINGAPORE 169610

Our Ref: 2269060/VPA/SJW

Re: New United Kingdom Patent Application
in the name of National Cancer Centre of Singapore Pte Ltd
"Novel Polynucleotide and Polypeptide and Uses Therefor"

Dear Dr Huynh

In accordance with standing instructions, we confirm that we forwarded instructions to our U.K. Associates, to file the above application on ***24 May 2000.*** You will receive official notification of the filing of this application form our Overseas Filing Department in due course.

In the meantime, please do not hesitate to contact us if you have any questions in this regard.

Yours sincerely
DAVIES COLLISON CAVE

VICTOR P ARGAET

cc Dr Nicholas Tay, National Cancer Centre of Singapore Pte Ltd,
11 Hospital Drive Singapore 169610

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DAVIES COLLISON CAVE
PATENT & TRADE MARK ATTORNEYS

12 May, 2000

Dr Hung Huynh
National Cancer Centre of Singapore Pte Ltd
11 Hospital Drive
SINGAPORE 169160

Our Ref: 2269349/VPA/aal

Re: New United Kingdom Patent Application
in the name of National Cancer Centre of Singapore Pte Ltd
"Novel compositions and methods of using them"

Dear Dr Huynh

In accordance with standing instructions, we confirm that the above application has been filed by our U.K. Associates. You will receive official notification of the filing of this application from our Overseas Filing Department in due course.

In the meantime, please do not hesitate to contact us if you have any questions in this regard.

Yours sincerely
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VICTOR PARGAET

cc Dr Nicholas Tay, National Cancer Centre Pte Ltd, 11 Hospital Drive, Singapore, 169610

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Appendix 7

DAVIES COLLISON CAVE
PATENT & TRADE MARK ATTORNEYS

12 May, 2000

Dr Hung Huynh
National Cancer Centre of Singapore Pte Ltd
11 Hospital Drive
SINGAPORE 169160

Our Ref: 2269336/EJH/aal

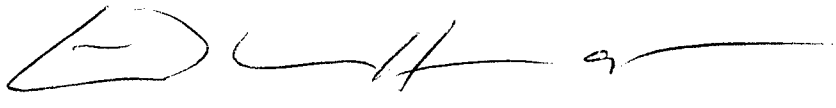
Re: New United Kingdom Patent Application
in the name of National Cancer Centre of Singapore Pte Ltd
"A nucleic acid molecule and uses therefor:

Dear Dr Huynh

In accordance with standing instructions, we confirm that the above application was filed by our U.K. Associates on ***3 May 2000***. You will receive official notification of the filing of this application from our Overseas Filing Department in due course.

In the meantime, please do not hesitate to contact us if you have any questions in this regard.

Yours sincerely
DAVIES COLLISON CAVE



E JOHN L HUGHES

cc Dr Nicholas Tay, National Cancer Centre Pte Ltd, 11 Hospital Drive, Singapore, 169610

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Appendix 8

Cloning and Characterization of a Novel Pregnancy-induced Growth Inhibitor in Mammary Gland

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Running Title: Cloning of a Mammary Gland Growth Inhibitor

Abstract

Growth factors and growth inhibitors play an important role in the regulation of growth and differentiation of mammary epithelial cells. It is well known that during pregnancy, with the onset of terminal differentiation, the proliferation of mammary epithelial cells dramatically decreases. We report the cloning and characterization of a novel pregnancy-induced cDNA (namely *OKL38*) from a human ovarian cDNA library. This cDNA encodes a protein of approximately 34.5 kDa. Northern blot analysis from various tissues revealed the distribution of *OKL38* transcripts in most tissues, with the highest level in the ovary, kidney and liver. *OKL38* gene expression gradually increases as pregnancy advances and was induced by human chorionic gonadotropin, which is known to induce mammary gland differentiation. Antibody raised against OKL38 recognized a 38 kDa in most of the tissues examined with the highest levels in the heart, cerebellum, kidney and liver. OKL38 transcripts were low in all human breast cancer cell lines examined and barely detectable in all DMBA-induced mammary tumours examined. Transfection of *OKL38* cDNA into MCF-7 cells resulted in growth inhibition and reduction in tumour formation *in vivo*. The above observations suggest that the *OKL38* may play a crucial role in the growth regulation and differentiation of the breast epithelial cells during pregnancy and tumorigenesis.

Introduction

Breast cancer is the most common cancer and the second cause of cancer death in women. Globally, the incidence of breast cancer appears to be increasing and an annual worldwide incidence of over one million is predicted by the turn of this century (1).

Epidemiological studies have demonstrated that for women early age at menarche, late age at first pregnancy and late age at menopause tend to increase the risk for breast cancer (2,3). The lifetime risk of breast cancer is 2 to 5 times higher in women who have a first pregnancy after age 30 than in women whose first pregnancy is at an age younger than 20 (2,3). It has been hypothesized that first pregnancy at a young age may differentiate breast cells early in life, after which they would become less susceptible to carcinogens (4-6). This hypothesis was supported by the observation that in animal models, mammary tumorigenesis is facilitated when the administration of carcinogen precedes pregnancy, however it decreases when the carcinogen exposure occurs during pregnancy (7). Normal and prolonged lactation in mice and in rats is also recognized to result in a decrease in the incidence of spontaneous or carcinogen-induced mammary tumours and an increase in tumour age when compared with forced breeding without lactation (7). In accordance, mammary DNA synthesis is at a very low level during lactation in mice and rats. These observations in experimental animals show protection of pregnancy and lactation against mammary tumorigenesis.

Both animals and humans are constantly exposed to carcinogenic agents during their life time. It

is conceivable, therefore, that the longer the total period of low mammary DNA synthesis, i.e., proliferative mitotic rest, owing to pregnancy, lactation, etc., the smaller is the risk of mammary malignancy. In human, only during the first trimester of pregnancy, breast parenchymal growth and DNA synthesis are pronounced. The latter half of pregnancy is the period of proliferative and mitotic rest and breast parenchyma shows only minor DNA synthesis. The protective effects of having children early in life may accrue by causing breast cells to become more differentiated. It is impractical to suggest early pregnancy as a breast cancer prevention strategy, but an investigation of the physiological basis of this protection may lead to novel risk reduction strategies.

Peptide growth factors and inhibitors play key roles in regulating the proliferation of normal breast epithelium (8). The importance of peptide growth factors in the pathogenesis and behaviour of breast neoplasms is evident in the large amount of literature that has accumulated in the past decade concerning the roles of EGF, IGFs, TGF-α, TGF-ß, and FGF(8,9). To date, the best characterized inhibitor is TGF-ß (10). A negative regulatory function for insulin-like binding protein 3 (11-14), and for mammary derived growth inhibitor (15) have been reported. Abnormal expression of growth factors and growth inhibitors has been implicated in tumorigenesis (15-17). These observations suggest that interruption of growth factor action (or production) or enhancement of growth inhibitor production by breast cancer cells would represent new strategies for arrest of tumour growth.

We report the cloning and characterization of a novel cDNA, OKL38. OKL38 gene expression

was induced by pregnancy and hormone associated with pregnancy such as hCG. OKL38 expression was low in breast cancer cell lines and barely detectable in DMBA-induced breast tumours. Transfection of human MCF-7 breast cancer cells with OKL38 cDNA lead to reduction in proliferation and tumour formation in nude mice. We conclude that OKL38 is a hormonally regulated gene with intriguing differentiating and antiproliferative properties for breast epithelial cells.

Materials and Methods

mRNA differential display: Differential display was performed using RNA from pregnant mammary gland according to the protocol supplied with the RNAmap^{TM} kit (GeneHunter Corp., Nashville, TN). Briefly, 5 μg of DNAse I-treated total RNA were reverse transcribed with $T_{12}M^n$ (where n may be G, A, T, or C), followed by PCR amplification in the presence of [α-^{33}P]dATP (NEN) using the corresponding $T_{12}M_n$ primer, downstream, and one arbitrary primers supplied with the kit, AP_1-AP_5, upstream. The PCR-amplified fragments were separated on 6% denaturing polyacrylamide gel. The gel was dried and exposed to Kodak XAR film, and cDNAs representing differential expressed mRNAs were excised from the dried gels and reamplified cDNA fragments were used to as probes in Northern blotting to verify their differential expression in mammary gland. The differential probe was used to screen human ovarian cDNA library as described (18). The isolated human cDNAs were sequenced by the Sanger dideoxy chain determination method and their nucleotide sequences were compared with those deposited in the Genbank and EMBL data banks.

Animals and drug administration. Animal experiments were approved by local Animal Care Committee. Female Sprague-Dawley rats, 50 days old at the beginning of the experiments, were obtained from Charles River, Quebec. To study changes in OLK38 expression during pregnancy and lactation of normal rats, pregnant rats were sacrificed on days 0, 4, 10, 16, and 21 of pregnancy and day 3 of lactation. Mating dates were established from the appearance of vaginal plugs. Day 1 of pregnancy was the day on which a plug was observed. The animals were

sacrificed and the mammary gland collected as described above.

To study the effects of human chorionic gonadotropin on OKL38 expression, rats were inoculated by i.p. injection with doses of 10, 20 and 40 UI of hCG/day in 200 μl of PBS for 21 days in a manner such as to simulate levels seen at time of lactation as described (19). Control rats were administered 200 μl PBS. After the last injection, the rats were allowed to rest for an additional 7 days and at the end of the experiment they were sacrificed. Breast tissue was assayed for OKL38 mRNA Northern blotting.

Induction of mammary tumours by DMBA. We used the standard DMBA-induced mammary tumour experimental model (20) to study the expression of OKL38 gene during pregnancy. Mammary carcinomas were induced by a single intragastric administration of 20 mg dimethylbenz(A)anthracene (DMBA, Sigma Chemical Co., St. Louis, MO) in 1 ml peanut oil at 50-52 days of age. This standard procedure yields palpable (> 0.5 cm) tumours in about 75% of animals by day 80 following carcinogen administration. Rats bearing DMBA-induced breast tumours were mated. Mating dates were established from the appearance of vaginal plugs. Pregnant rats were sacrificed on day 16 of pregnancy and tumours were collected.

To determine the OKL38 gene expression in human breast cancer cell lines, MCF-7 , T47D, ZR75, MDA-231, Hs578T, and HBL-100 were grown to 90% confluence. Poly "A" RNA was purified and Northern blotting was performed to determine the levels of OKL38 mRNA.

MCF-7 cell stable transfectant cell lines: The entire coding region of OKL-38 cDNA was cloned into mammalian expression vector pcDNA3.1. MCF-7 cells were seeded at 2x 10^5 in 100 mm culture dishes in 90% α-MEM (Life Technologies, Inc.) containing 10% FCS with Garamycine 24 h prior to transfection. Cells were transfected with 5 μg of full-length OKL38 cDNA (pcDNA3.1-OKL38) or pDNA3.1 control plasmid DNA and 28 μl of Lipofectamine reagent (Life Technologies) following recommendations of the manufacturer. 48h following transfection, cells were split 1:10 and replaced with growth medium containing 800 μg/ml G418 (Calbiochem, La Jolla, CA). After 4 weeks, clones were isolated, expanded and assayed for OKL38 expression by Western and Northern blot analyses.

Cell number refers to mean cell number counted by hemocytometer 8 days after seeding 2.5x10^4 cells in wells containing α-MEM supplemented with 10% fetal calf serum. Means were determined from quadruplicate replicate wells and in no case did standard deviation exceed 15% of the mean value.

In vivo tumor formation was assayed using 4-8 week old athymic nude mice (CD1 nu/nu, Charles River). Each cell line was assayed in four mice, and each mouse received an injection of 5x10^6 cells into an inframammary fat pad, and another identical injection of the same cell line into a contralateral fat pad. 30 days after injection, animals were inspected for grossly visible tumours.

Statistical analysis: Differences in OKL38 gene expression were analysed by Student's t-test. Differences in cell number and tumor number between parental lines and transfectants were

tested using the Mann Whitney U-test.

Generation of an OKL38 antibody- Synthetic peptides corresponding to predicted amino acids 243 to 267 of the human OKL38 (N- **arg glu gln ser ile leu ser pro ser pro tyr glu gly tyr arg ser leu pro arg his gln leu leu cys phe** -C) were synthesised and coupled to preactivated keyhole limpet hemocyanin (Sheldon Biotechnology, Montréal, Quebéc). Rabbit polyclonal antibodies were produced according to standard protocols. Affinity purified serum following the sixth boost was used in these studies.

Western Analysis: Mammary tissue were homogenized in buffer containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1% NP-40, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μM PMSF, and 100 μM $NaVO_4$. Cells were lysed in the above buffer. Cell lysate was used to determine changes in the levels of OKL38 by Western blottings were as described (21). Blots were incubated with rabbit anti-OLK38 (1:500 dilution) and horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody (1:7500). Blots were visualized with a chemiluminescent detection system (ECL, Amersham) and exposed to film for 10 sec to 45 sec.

Northern Blot: Poly "A" RNA was isolated from indicated tissues of female rats as described (22). Northern blots were performed on poly "A" RNA or total RNA and blots were hybridized with human OKL38 or human GAPDH (ATCC) cDNAs as previously described (22). mRNA levels were determined by densitometric scanning of autoradiograms.

Results

RNA derived from mammary tissue of non-pregnant and pregnant rats was subjected to differential display. Among 18 diffrential bands obtained, one band of approximately 450 bp was novel as determined by sequence analysis. The differential expression of this particular cDNA was confirmed by Northern blotting. The 450 bp probe detected an approximate 1.6 kb mRNA species in the human, rat and mouse mammary tissues (data not shown).

In order to clone the full length human cDNA, human ovarian cDNA library was screened using this 450 bp probe. Eight positive clones were isolated and clone-purified. One clone containing an approximate 1.6 kb insert was isolated and sequenced by the Sanger dideoxy chain determination method.

Comparison of the nucleotide sequence against the non-redundant nucleotide database of Genebank established this 1.6 kb cDNA was novel. Blast search revealed no significant homology with any known sequences. This cDNA (Genebank accession no AF191740) contained 1607 bp and was full length cDNA. An initiator ATG codon (position 127) is followed by a single open reading frame of 317 amino acids with a calculated molecular weight of 34.5 kDa. The open reading frame ended with a TGA terminator codon at position 1078 followed by 529 nucleotides in the 3' untranslated region.

To determine the distribution of 1.6 kb transcripts, Northern blot analysis was performed using

poly "A" RNA derived from various tissues of mature female rats. The band of approximate 1.6 kb were observed in all tissues with the highest levels were seen in the ovary, kidney and liver (Fig. 1). Minor bands of approximate 3.6 and 4.0 kb were also observed. Western blotting using rabbit polyclonal antibody against OKL38 protein recognized a 38 kDa protein in most of the tissues examined with the highest levels found in the heart, cerebellum, kidney and liver (Fig. 2). We named this protein is OKL38.

To investigate the changes of OKL38 expression during pregnancy and lactation, poly "A" RNA and proteins derived from mammary gland at different stages of pregnancy were analysed by Northern and Western blottings, respectively. As shown in figure 3, the levels of OKL38 mRNA were very low in mammary tissue of non-pregnant rats. Following pregnancy, the OKL38 mRNA increased rapidly and maximal OKL38 expression was observed during lactation. Western blot analysis revealed that OKL38 protein already peaked during early pregnancy and remained throughout pregnancy and lactation (Fig. 3D).

To determine if human chorionic gonadotropin (hCG), a known mammary differentiating agent, was capable to increase OKL38 gene expression, female rats were injected with hCG in a manner such as to simulate levels seen at time of lactation (19). Figure 4 shows that hCG induced OKL38 gene expression in a dose-dependent manner.

To dertermine the OKL38 gene expression in human breast cancer cells, poly "A" RNA derived from various cell lines was analysed by Northern blot analysis. Figure 5 shows that although the

OKL38 transcripts were detected in all breast cancer cell lines examined, the mRNA levels were about 10 to 15% of that seen in breast tissue. Despite detectable OKL38 mRNA, OKL38 protein in these cell lines was barely detected as determined by Western blotting (data not shown).

Since OLK38 is highly expressed in breast tissue during pregnancy, we undertook the first study of OKL38 expression in neoplastic tissue, using the rat DMBA-induced mammary tumour experimental system (20). Figure 6 shows the results of an experiment where we first exposed animals to DMBA, allowed tumours to appear, and then allowed the rats to become pregnant. In lactating rats bearing DMBA-induced tumours, the OKL38 expression seen in normal mammary gland was abundant relative to that seen in most mammary ductal neoplasms. OKL38 proteins shared similar pattern as determined by Western blotting (data not shown).

To test the hypothesis that the gene encoding OKL38 is a tumor suppressor gene, human MCF-7 breast cancer cells were transfected with an expression vector for full-length OKL38 cDNA (pcDNA3.1-OKL38). Figure 7 shows high levels of expression of OKL38 mRNA (~1.6 kb) in representative transfected cell lines SQ13 and SQ18, but the absence of expression in untransfected MCF-7 and the mock-transfected cells. Western blotting with a polyclonal anti-OKL38 antiserum was used to detect OKL38-related protein in the various clones. A 38 kDa protein was detected only in cells transfected with pcDNA3.1-OKL38 (Fig. 7C).

Proliferative behaviour of OKL38-expressing clones was evaluated by determining *cell number* on plastic dishes after 8 days of incubation. The number of cells was significantly less ($p < .05$, Mann-

Whitney U-test) in OKL38-expressing transfectants than in controls (Fig. 7D).

To determine if over-expression of OKL38 leads to reduction in tumour formation, *in vivo* tumorgenicity was performed. As shown in figure 7E, the rate of tumor formation following injection of MCF-7 cells was 100% (8 of 8), that of pcDNA3.1-1 mock-transfected cells 88% (7 of 8), and that of SQ13 and SQ18 transfected cells 12.5% (1 of 8) and 25% (2 of 8), respectively.

Discussion

Studying the pregnant associated with breast cancer protection, we isolated a pregnancy-induced growth inhibitor cDNA, OKL38, using a differential display. Comparison of the nucleotide sequence obtained against the non-redundant nucleotide data base of GeneBank established that OKL38 cDNA was novel and shared no signinicant homology with any published sequences. The OKL38 cDNA contains 1607 bp and encodes 317 amino acids with a calculated molecular weight of 34.5 kDa.

The human OKL38 cDNA and its antibody detected OKL38 mRNA and protein, respectively derived from rat and mouse tissues suggesting that OKL38 gene is conserved among human, rat and mouse. In order to determine the homology among rat, human and mouse OKL38 cDNA, experiments are underway to clone the rat and mouse OKL38 cDNAs.

Tissue survey revealed that OLK38 transcripts were detected in all tissues examined with the highest levels in the ovary, kidney and liver. High molecular weight mRNAs were also detected. It is unknown whether these transcripts are precursor of mature transcript or different transcripts arrived as a result of differential splicing. Western blot analysis revealed that anti OKL38 recognized a 38 kDa protein which were abundant in heart, cerebellum, kidney and liver. Despite low levels of OKL38 mRNA in the heart and cerebellum (Figure 2), OKL38 protein content was high in these tissues suggesting that translational and post-translational controls of OKL38 protein in these tissues.

The gene expression of OKL38 was low in MCF-7 cells and other human breast cancer cell lines examined. Furthermore, OLK38 immunoreactivity is barely detectable in these cells. It is possible that loss of OKL38 production is required for changes from normal to malignant state and also confers the growth avantage over normal cells. Increasing OKL38 levels following transfection lead to a reduction in cellular growth and tumour formation in nude mice suggesting OKL38 plays an important role in growth regulation and tumorigenesis. The demonstration of tumour suppressor activity in MCF-7 cells is provocative, but to support the hypothesis that OKL38 is unrelated to characterized growth inhibitory or tumor suppressor protein. The observed tumor suppressor activity of the OKL38 gene is comparable to that previously documented using similar assays for Rb, p53, and H19 (23-26).

The abundance of OLK38 transcripts in poly "A" RNA extracted from the entire mammary gland is low. The expression is significantly increased above baseline at the time of the physiological changes associated with pregnancy and lactation (specifically maximal breast epithelial differentiation). In *in vivo* experiments of normal breast, we have evidence for induction of OKL38 expression by hCG. These observations document the existence of hormonal regulation of OKL38 expression. A strong relationship between onset of differentiation, inhibition of proliferation, and onset of OKL38 expression is observed. This observation suggests that a novel approach to breast cancer prevention, at least in certain populations, would therapeutically use hCG to encourage OKL38 expression to lower breast cancer risk. This approach would in fact represent a mimicking of the well-known protective effect of early pregnancy on subsequent cancer: the terminal

differentiation associated with lactation has been proposed as a mechanism underlying the protective effect of pregnancy (19). This information may give insight into novel actions of hCG that may be related to its differentiation and antineoplastic activity (19).

The absence of a detectable signal in the neoplastic tissue is of particular interest. In this tissue, the hormonal changes associated with pregnancy are associated with upregulation of OKL38 expression *in vivo,* but this normal induction of expression does not take place in neoplastic breast tissue. It is possible that the neoplastic progression itself is associated with reduction in OKL38 expression. The molecular mechanisms responsible for OKL38 inactivation in DMBA-induced breast tumours were not known. It is possible that OKL38 expression may absent from mammary tumors as a consequence of genetic alterations such as deletion, mutation, or inappropriate hypermethylation (27-29). Experiments are underway to determine the mechanisms responsible for silencing OKL38 expression in breast tumours.

At the present time, the functional significance of the OKL38 proteins in liver, heart, cerebellum and kidney is unknown, but it is intriguing that breast seems to be a tissue in which OKL38 expression varies greatly according to differentiation state (which of course varies considerably in this organ according to development, lactation, etc.), in contrast to stable expression of OKL38 protein in other organs characterized by relatively low cellular turnover and extensive differentiation (eg. kidney, cerebellum, liver and cardiac muscle). It appears that post-transcriptional controls of OKL38 expression play an essential role in determining the levels of OKL38 protein in these tissues.

In summary, our data indicate that OKL38 is a growth inhibitor and tumour suppressor. OKL38 expression is upregulated by pregnancy and hormone that induce differentiation such as hCG. The expression of OKL38 is associated with differentiation and low proliferative rate. Additional knowledge in the growth inhibitory and antitumour activity of OKL38 protein may be relevant to understanding the basis of the protective effect of pregnancy on subsequent cancer risk (2,3). The data suggest that enhancement of OKL38 production by breast cancer cells would represent new strategies for arrest of tumour growth.

Figure Legends

Figure 1. Northern blot analysis of OKL38 gene expression in adult rat tissues. Two μg of poly "A" RNA derived from each tissue of 3 months old rat was subjected to Northern blot analysis. Blots were hybridized with GAPDH (**A**) and human OKL38 (**B**) cDNAs. Densitometric scanning of the 1.6 kb band is shown in (**C**). Tissues are: **He**: heart; **Ut**: uterus; **Lu**: lung; **Ov**: ovary; **Ce**: cerebellum; **Pr**: prostate; **Ki**: kidney; **Sp**: spleen; **Pi**: pituitary; **In**: small intestine; **Mu**: red muscle; **Li**: liver; and **Mg**: mammary gland. Highest levels of OKL38 mRNA were detected in ovary, kidney and liver. High molecular OKL38 transcripts were also detected.

Figure 2. Western blot analysis of OKL38 protein in adult rat tissues. Total proteins extracted from various tissues of 3 months old rat was analysed by Westhern blotting. Blots were incubated with rabbit polyclonal anti OKL38 and mouse anti α-tubulin antibodies. Tissues are: **Cer**: cerebellum; **Pi**: pituitary; **Ut**: uterus; **Ov**: ovary; **Mg**: mammary gland; **He**: heart; **Bla**: bladder; **Spl**: spleen; **Int**: small intestine; **Fa**: fat; **Li**: liver; **Lu**: lung; **Ki**: kidney; **Mu**: red muscle. Highest levels of OKL38 Protein were observed in heart, kidney, liver and cerebellum.

Figure 3. Changes in OKL38 expression in the mammary gland during pregnancy and lactation. Two μg of poly "A" RNA derived from mammary gland on days 0 (Lane **1**), 4 (Lane **2**), 10 (Lane **3**), 16 (Lane **4**), 21 (Lane **5**) of pregnancy and day 3 of lactation (Lane **6**) were subjected to Northern blotting. Blots were hybridized with human OKL38 (**A**) and GAPDH (**B**) cDNAs. Densitometric scanning of the 1.6 kb band is shown in (**C**). OKL38 transcripts increased following

pregnancy and lactation. (**D**) Detection of OKL38 protein in the mammary gland during pregnancy and lactation.

Figure 4. Effects of human chrorionic gonadotropin on OKL38 gene expression. Female rats were treated with indicated concentrations of hCG for 3 weeks. Two μg of poly "A" RNA derived from mammary tissues were subjected to Northern blotting. Blots were hybridized with human OKL38 (**A**) and GAPDH (**B**) cDNAs. Densitometric scanning of the 1.6 kb band is shown in (**C**). Note that OKL38 gene expression was significantly induced by hCG ($p<0.01$) which is known to induce mammary gland differentiation.

Figure 5. Detection of OKL38 transcripts in human breast cancer cell lines. Two μg of poly "A" RNA derived from human breast cancer cell lines were subjected to Northern blotting. Blots were hybridized with human OKL38 (**A**) and GAPDH (**B**) cDNAs. Positive control mammary gland RNA (Lane **1**) and cell lines: MCF-7 (Lane **2**); T47D (Lane **3**); ZR75 (Lane **4**); MDA-231 (Lane **5**); Hs578T (Lane **6**) and HBL-100 (lane 7). Note that OKL38 gene expression was low in all human breast cancer cell lines.

Figure 6. OKL38 gene expression in the mammary gland during pregnancy and DMBA-induced breast tumours. Two μg of poly "A" RNA derived from normal mammary tissues of pregnant rats or DMBA-induced mammary tumours of pregnant rats were analysed by Northern blotting. Blots were hybridized with ß-actin (**A**) and OKL38 (**B**) cDNAs. In pregnant rats bearing DMBA-induced tumours, the OKL38 expression seen in normal mammary gland is abundant

relative to that seen in all mammary ductal neoplasms.

Figure 7. Effects of stable transfection of breast cancer cell line with OKL38 cDNA. Northern blot hybridization with a [^{32}P]dCTP-labeled OKL38 probe (**B**) of total RNA (50 μg/lane) extracted from parental MCF-7 cells (**P**), pcDNA3.1 vector transfected cell lines (lines **1**, **2** and **34**) and OKL38 transfectants (**SQ13** and **SQ18**). RNA loading amounts were compared by ethidium bromide staining of 18S and 28S rRNA (**B**). Immunodetection of 38 kDa OKL38 in stably transfected cell lines SQ13 and SQ18 (**C**). Proliferative behaviour of the clones expressing OKL38 by determining *cell number* on plastic dishes after 8 days incubation (**D**). The number of cells was significantly less ($p < .05$, Mann-Whitney U-test) in OKL38-expressing transfectants than in controls. Tumour formation of stably transfected cell lines SQ13 and SQ18 (**E**). The rate of tumor formation was significantly less ($p < .05$, Mann-Whitney U-test) in OKL38-expressing transfectants than in controls.

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       130         140         150         160         170         180
GACTACCTGT CCGAAGGCCT CGAAGGCCGA TCCCAAAGCC CCGTGGCCCT GCTCTTTGAT
       190         200         210         220         230         240
GCCCTTCTAC GCCCAGACAC AGACTTTGGG GGAAACATGA AGTCGGTCCT CACCTGGAAG
                                            M   K   S   V   L   T   W   K

       250         260         270         280         290         300
CACCGGAAGG AGCACGCCAT CCCCCACGTG GTTCTGGGCC GGAACCTCCC CGGGGGAGCC
 H   R   K   E   H   A   I   P   H   V   V   L   G   R   N   L   P   G   G   A
10                                          20

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 W   H   S   I   E   G   S   M   V   I   L   S   Q   G   Q   W   M   G   L   P
30                                          40

       370         380         390         400         410         420
GACCTGGAGG TCAAGGACTG GATGCAGAAG AAGCGAAGAG GTCTTCGCAA CAGCCGGGCC
 D   L   E   V   K   D   W   M   Q   K   K   R   R   G   L   R   N   S   R   A
50                                          60

       430         440         450         460         470         480
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 T   A   G   D   I   A   H   Y   Y   R   D   Y   V   V   K   K   G   L   G   H
70                                          80

       490         500         510         520         530         540
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 N   F   V   S   G   A   V   V   T   A   V   E   W   G   T   P   D   P   S   S
90                                         100

       550         560         570         580         590         600
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 C   G   A   Q   D   S   S   P   L   F   Q   V   S   G   F   L   T   R   N   Q
110                                        120

       610         620         630         640         650         660
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 I   G   A   G   L   S   A   A   D   A   V   L   Y   A   R   H   Y   N   I   P
190                                        200

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 V   I   H   A   F   R   R   A   V   D   D   P   G   L   V   F   N   Q   L   P
210                                        220

       910         920         930         940         950         960
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 K   M   L   Y   P   E   Y   H   K   V   H   Q   M   M   R   E   Q   S   I   L
230                                        240

       970         980         990        1000        1010        1020
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 S   P   S   P   Y   E   G   Y   R   S   L   P   R   H   Q   L   L   C   F   K
250                                        260
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 E  D  C   Q  A  V  F   Q  D  L   E  G  V   E  K  V  F   G  V  S
270                             280
      1090        1100        1110        1120        1130        1140
CTGGTGCTGG TCCTCATCGG CTCCCACCCC GACCTCTCCT TCCTGCCTGG GGCAGGGCTG
 L  V  L   V  L  I  G   S  H  P   D  L  S   F  L  P  G   A  G  L
290                             300
      1150        1160        1170        1180        1190        1200
ACTTTGCAGT GGATCCTGAC CAGCCGCTGA GCGCCAAGAG GAACCCCATT GACGTGGACC
 T  L  Q   W  I  L  T   S  R  *
310
      1210        1220        1230        1240        1250        1260
CCTTCACCTA CCAGAGCACC CGCCAGAGGG CCTGTACGCC ATGGGGCCGC TGGCGGGGAC
      1270        1280        1290        1300        1310        1320
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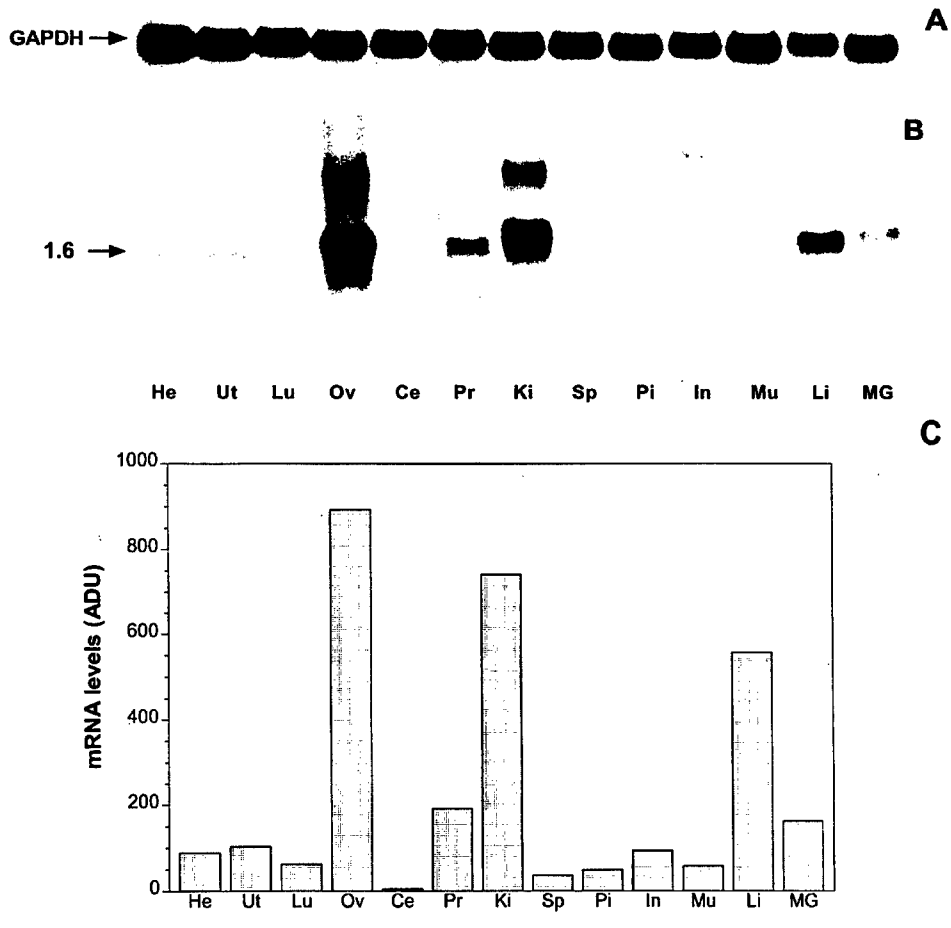
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B
1.6
He Ut Lu Ov Ce Pr Ki Sp Pi In Mu Li MG
C
mRNA levels (ADU)
1000
800
600
400
200
0
He Ut Lu Ov Ce Pr Ki Sp Pi In Mu Li MG

FIGURE 1

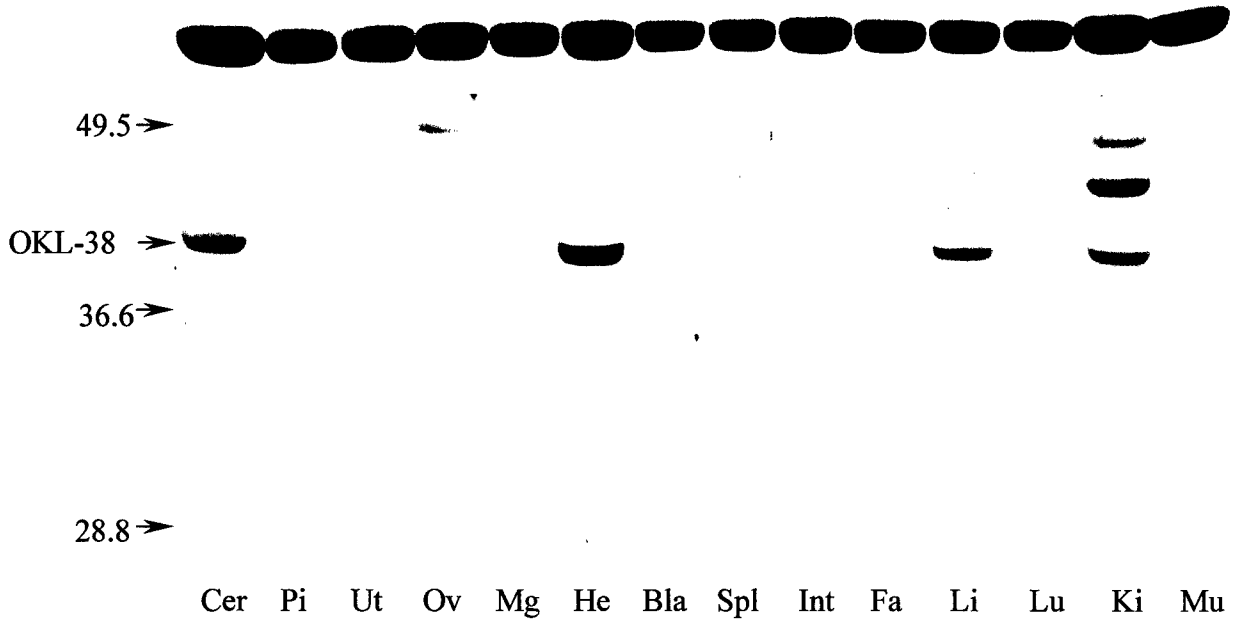
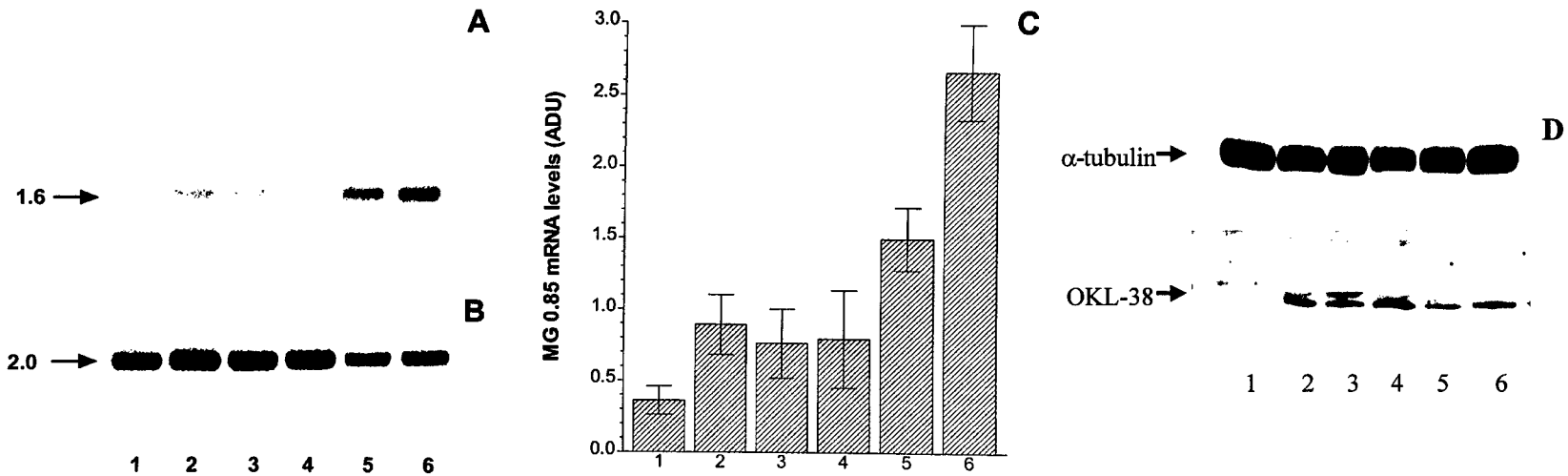


FIGURE 2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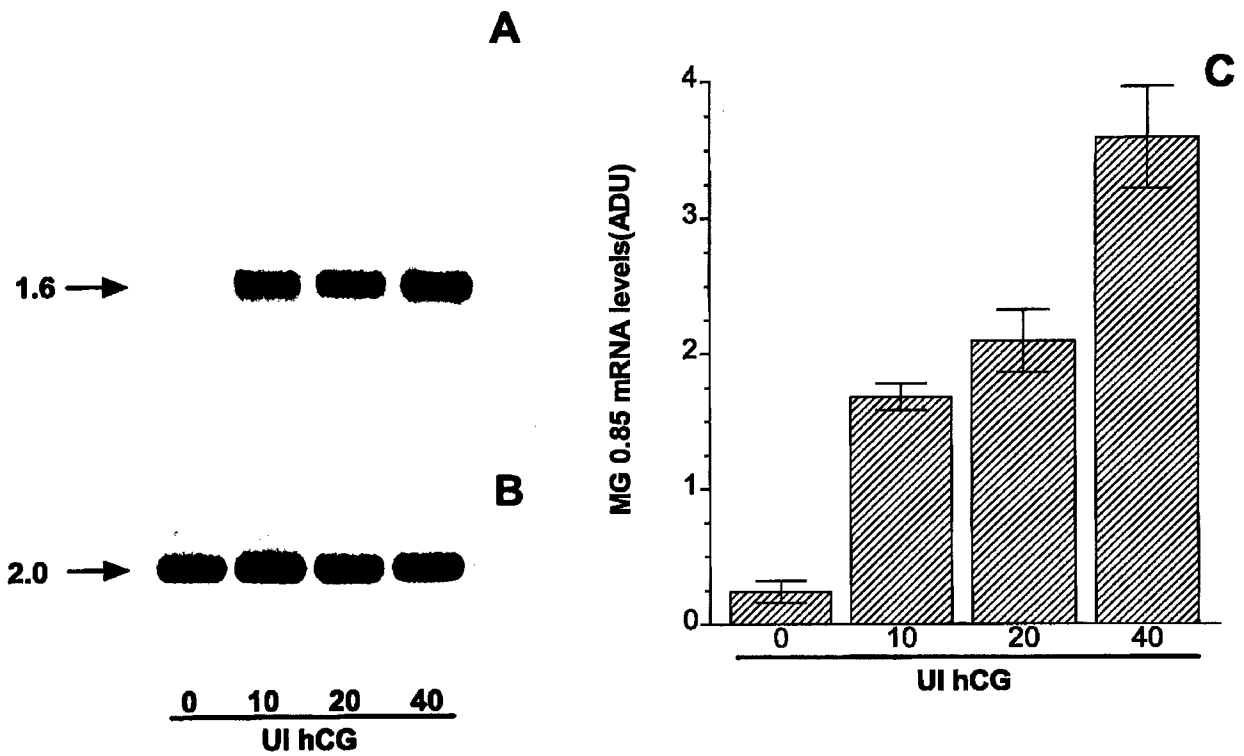
1.6
B
2.0
1 2 3 4 5 6
C
MG 0.85 mRNA levels (ADU)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1 2 3 4 5 6
D
α-tubulin
OKL-38
1 2 3 4 5 6

FIGURE 3


A
1.6
B
2.0
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C
MG 0.85 mRNA levels(ADU)
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2
3
4
0 10 20 40
UI hCG

FIGURE 4

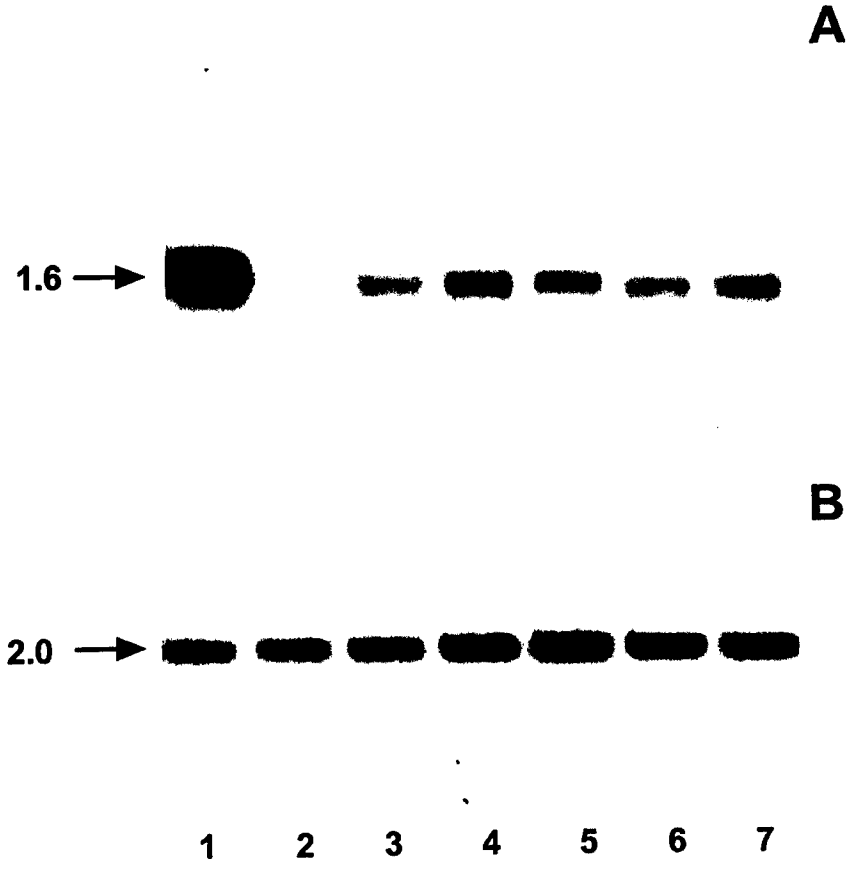


FIGURE 5

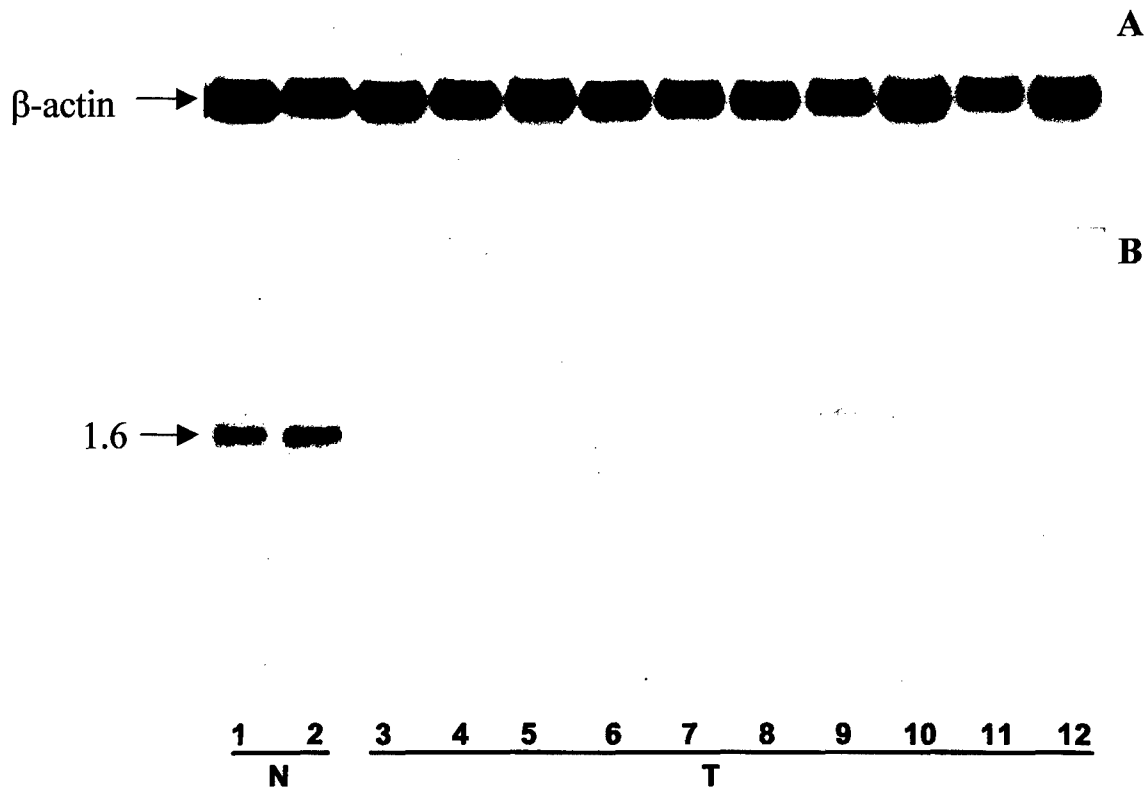
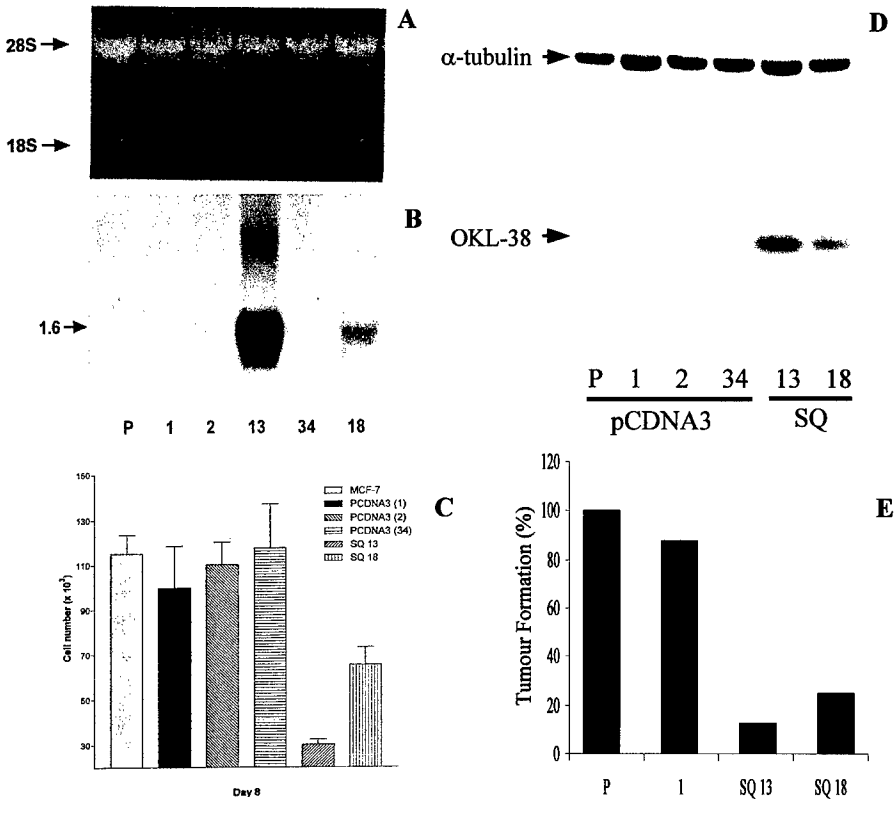


FIGURE 6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18S
B
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PCDNA3 (1)
PCDNA3 (2)
PCDNA3 (34)
SQ 13
SQ 18
Cell number (x 10³)
Day 8
D
α-tubulin
OKL-38
P 1 2 34 13 18
pCDNA3
SQ
E
Tumour Formation (%)
P
1
SQ 13
SQ 18

FIGURE 7

Induction of mammary epithelial cell differentiation and inhibition of dimethylbenz(A)anthracene-induced mammary tumour by co-administration of a pure anti-estrogen ICI 182780 and testosterone enanthate

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Running Title: Mammary gland differentiation and tumour suppression by antiestrogen and testosterone

Key words: mammary, differentiation, tumour, anti-estrogen, testosterone

Abstract

Epidemiological studies have shown that early first pregnancy is associated with a life-long reduction in breast cancer risk. The terminal differentiation associated with pregnancy and lactation has been proposed as a mechanism underlying the protective effect of pregnancy. We report that treatment of rats with ICI 182780 (ICI) caused a marked reduction in epithelial cells and Ki-67 labelling index as compared to controls and testosterone enanthate-treated (TE) mammary glands. TE increased the Ki-67 labelling index, stimulated lobuloalveolar and ductal growth, as well as the secretory activity of acinar cells. Co-administration of TE and ICI resulted in a reduction in Ki-67 labelling index. Mammary epithelial cells became differentiated, resembling that observed at the end of pregnancy and during lactation as indicated by marked increase in secretory activity, lipid accumulation and presence of basal nuclei. The expression of differentiation markers such as whey acidic protein, mammary derived growth inhibitor, α-casein and ß-casein was dectected only in TE plus ICI treated mammary tissues. Unlike TE, ICI caused a significant reduction in DMBA-induced tumour incidence, number of tumour bearing and tumour size. Tumour incidence was reduced to 8% when both ICI and TE were co-administered. Our data provide the novel molecular interactions between the estrogen and androgen in regulation of mammary growth and differentiation. These observations may give insight into novel actions of ICI and TE on breast differentiation and protection against carcinogenesis which may be useful in designing novel strategies for cancer prevention and/or treatment based on maximising mammary epithelial cell differentiation.

Introduction

Breast cancer is the most common cancer and the second cause of cancer death in women. Approximately one in nine North American women living to 80 years will develop breast cancer (1). Globally, the incidence of breast cancer appears to be increasing and an annual world-wide incidence of over one million is predicted by the turn of this century (2).

Epidemiological studies have demonstrated that for women early age at menarche, late age at first pregnancy and late age at menopause tend to increase the risk for breast cancer (3,4). The lifetime risk of breast cancer is 2 to 5 times higher in women who have a first pregnancy after age 30 than in women whose first pregnancy is at an age younger than 20 (3,4). It has been hypothesized that first pregnancy at a young age may differentiate breast cells early in life, after which they would become less susceptible to carcinogens (5-8). This hypothesis was supported by the observation that in animal models, mammary tumorigenesis is facilitated when the administration of carcinogen precedes pregnancy, however it decreases when the carcinogen exposure occurs during pregnancy (8). Normal and prolonged lactation in mice and in rats is also recognized to result in a decrease in the incidence of spontaneous or carcinogen-induced mammary tumours and an increase in tumour age when compared with forced breeding without lactation. In accordance, mammary DNA synthesis is at a very low level during lactation in mice and rats. These observations in experimental animals show protection of pregnancy and lactation against mammary tumorigenesis.

Both animals and humans are constantly exposed to carcinogenic agents during their life time. It is conceivable, therefore, that the longer the total period of low mammary DNA synthesis, i.e.,

proliferative mitotic rest, owing to pregnancy, lactation, etc., the smaller is the risk of mammary malignancy. In human, only during the first trimester of pregnancy, breast parenchymal growth and DNA synthesis are pronounced. The latter half of pregnancy is the period of proliferative and mitotic rest and breast parenchyma shows only minor DNA synthesis (9). The protective effects of having children early in life may accrue by causing breast cells to become more differentiated. Differentiation restricts the ability of a cell to grow abnormally, change its type and survive in other types of tissues. It is impractical to suggest early pregnancy as a breast cancer prevention strategy, but an investigation of the physiological basis of this protection may lead to novel risk reduction strategies.

In women, a high proportion of primary breast cancers possess the estrogen receptor (ER) and require estrogen or estrogenic activities for tumour growth. Current therapies have been directed toward interruption of estrogen by oophorectomy or the use of antiestrogens (10-12). The antiestrogen drug tamoxifen improves the survival of women with breast cancer, and has proven to be clinically useful for the treatment of metastatic ER-positive tumours (10,11,13). The pure antiestrogen ICI 182780 (ICI) (12) exhibits effects on some patients following disease progression on tamoxifen (10). ICI has recently been shown to act as a growth inhibitor even in the complete absence of estrogen stimuli (14,15), and to actively regulate gene expression in a direction opposite to that of estrogens (15) and lacks tamoxifen's uterine side effects (16).

Androgen receptors are found in 85% of all breast cancer specimen investigated (17). Androgens have been used for the treatment of breast cancer in women (18). Antiproliferative effects of androgens on the growth of certain breast cancer cell lines have been reported (17). In addition, DHEA exerts a potent inhibitory effect on the development of DMBA-induced

mammary carcinoma in the rat (19,20). It has been shown that combination of estrogen and testosterone can induce a high incidence of breast cancer (21).

Our objective is to develop a novel approach to breast cancer prevention and/or treatment by therapeutically using physiological signals that encourage terminal differentiation to lower breast cancer risk. This approach would in fact represent a mimicking of the well-known protective effect of early pregnancy on subsequent cancer. We reported the potential benefits of combining the pure antiestrogen ICI 182780 and testosterone enanthate on growth, differentiation and the development of mammary carcinoma induced by DMBA in the rat.

Materials and Methods

Antiestrogen ICI 182780 and testosterone enanthate treatments: Animals were maintained and treated according to the guidelines of the Animal Care. The experimental protocol was approved by the Institution Animal Care Committee. Fifty day old female Sprague-Dawley rats (Charles River, Montreal, Quebec) were divided into 4 groups (12 animals per group). On the first day of the experiment, the animals of the appropriate groups were underwent isoflurane-induced anaesthesia. Animals were implanted with 0.5 cm silastic tube (0.125 in (outer diameter), and 0.062 in (inner diameter), Dow Corning, Midland, MI) either empty or containing testosterone enanthate (TE) (Sigma) inserted sc in the dorsal area of each animal of indicated groups. The animal were treated for 3 weeks with the following: 1) empty silastic tube and castor oil; 2) testosterone enanthate and castor oil; 3) empty silastic tube and preformulated ICI 182780 in castor oil (Zeneca Pharmaceuticals, England) at a dose of 1 mg/kg BW, once per

week; and 4) ICI (1 mg/kg/week) plus TE (1.2 μg/day). The dose of ICI 182780 was based on our previous studies. The rate of TE released per day was 1.2 μg. The proliferative marker Ki-67 and differentiative markers whey acidic protein (WAP), mammary derived growth inhibitor (MDGI), α- and β-caseins were used as surrogate measure of treatment success.

Induction of mammary tumours by DMBA. We used the standard DMBA-induced mammary tumour experimental model (22,23) to study the effects of TE and ICI on DMBA-induced tumour incidence, tumour size and tumour number. Mammary carcinomas were induced by a single intragastric administration of 20 mg dimethylbenz(A)anthracene (DMBA, Sigma Chemical Co., St. Louis, MO) in 1 ml peanut oil at 50-52 days of age. Rats were divided into 4 groups (n=20) and treated as follows 1) empty silastic tube and castor oil; 2) TE and castor oil; 3) empty silastic tube and ICI 182780 in castor oil (Zeneca Pharmaceuticals, England) at a dose of 1 mg/kg BW, once per week; and 4) ICI (1 mg/kg/week) plus TE (1.2 μg/day) one day before the oral administration of DMBA. Tumour size and number were recorded. The two largest perpendicular diameters of each tumour were recorded with callipers to estimate tumour size. Rats were killed 150 days after DMBA administration. The uteri, ovaries and mammary tissue were removed for later analysis. Analysis of the incidence of development of mammary tumours was performed using the Fishers exact test (24). The data are presented as the mean ± SEM.

Northern Blot: To investigate the effects of testosterone enanthate and ICI 182780 on the gene expression of milk protein genes and MDGI, total RNA was isolated from tissues as described (25). Northern blots were performed on total RNA and blots were hybridized with mouse α-casein, mouse β-casein, mouse WAP and mouse MDGI (26). To control for equal loading of wells, blots will be rehybridized with GAPDH cDNA (ATCC). Quantitative analysis of gene expression is accomplished by scanning autoradiograms densitometrically. The figure was adjusted for minor differences in the amount of RNA loaded.

Immunohistochemistry and histology: mammary tissue was immersed in a solution of 10% buffered formalin. After fixation, the mammary tissue was routinely processed in a tissue processor and embedded in paraffin. Sections of 5 μm were cut and stained with haematoxylin-eosin. Examination of the slides were performed by light microscopy. The ImmunoCruz Staining System was used for immunohistochemical study. Briefly, the slides were deparaffinized, rehydrated in water and incubated with 3% H_2O_2 for 20 min to block endogenous peroxidase activity. To examine expression of Ki-67, antigens were retrieved by heating the slides in citrate buffer (pH 6) for 5 min. After preincubation with normal serum for 20 min at room temperature, the primary antibody was applied (2 μg/ml) and incubated overnight at 4 ^{0}C. The section then incubated with the appropriate biotinylated secondary antibody at 1:500 dilution followed by peroxidase-conjugated streptavidin complex according to the manufacturer's instruction and DAB. The section then counterstained with haematoxylin. To evaluate the Ki-67 labelling index, in each group of 500 epithelial cells were counted in randomly chosen fields at x 400 magnification. The Ki-67 labelling index was expressed as the number of clearly labelled Ki-67 reactive nuclei in 500 cells counted. Significance difference was determined by Student's t-test.

Results

Control mammary gland had a sparse cluster of epithelial tubules surrounded by a small amount of connective tissue which was in turn embedded in a large fat pad. The epithelial ducts had a narrow, small lumen, lined by cuboidal cells with dark stained nuclei. The acinar cells were small and apparently inactive (Fig. 1A). Treatment of intact animals with ICI 182780 resulted in a marked atrophy of the mammary gland (Fig. 1B). The ICI-induced pattern was characterized by a decreased size of the lobular structures, which consisted of small atrophic alveoli, lined by atrophic and low cuboidal cells. The acinar epithelial cells were apparently inactive, with diminished quantity of cytoplasm (Fig. 1B). Testosterone enanthate stimulated lobuloalveolar and ductal growth, as well as the secretory activity of acinar cells. The lobuloalveolar structures consisted of hypertrophic acinar epithelial cells mainly filled with eosinophilic and, to a lesser degree, clear secretory vacuoles (Fig. 1C). Mammary ducts were only locally, mildly dilated. Administration of both TE and ICI, a significant increase in lobuloalveolar tissue of the mammary gland was seen (Fig. 1D). The lobular hyperplasia observed was characterized by size and number of the lobular structures. The lobuloalveolar units consisted of groups of alveoli lined by hypertrophic epithelial cells with highly eosinophilic cytoplasm filled mainly with eosinophilic secretory vacuoles similar to that observed in the mammary gland at the end of pregnancy (Fig. 1D).

We determined the effect of hormonal manipulation on Ki-67 expression in mammary tissue. Figure 2 shows the results of an experiment where mammary tissues were collected from rats treated with vehicle, ICI, TE, and ICI plus TE for immunohistochemical analysis of Ki-67 expression. TE significantly increased ($P<0.01$) while ICI significantly decreased ($P<0.01$) the

number of epithelial cells expressed Ki-67 as compared to controls. Further reduction in Ki-67 labelling index of the epithelium was seen when ICI and TE were co-administered as compared with the effect of each treatment alone ($P<0.01$).

Since whey acidic protein (WAP), α-casein, and β-casein and MDGI are hormonally regulated in rat breast epithelial cells and maximally expressed at the time of maximal differentiated function (26), the levels of WAP, α-casein, and β-casein and MDGI transcripts provide good markers for breast differentiation. As shown in Figure 3, the WAP, α-casein and β-casein transcripts were barely detected in control, ICI- and TE- treated mammary glands. Co-administration of TE and ICI resulted in a significant induction in WAP, α-casein and β-casein gene expression.

To determine whether changes in the mammary gland by TE plus ICI treatment resulted in a decrease in the incidence of carcinogen-induced mammary tumours, the DMBA-induced breast carcinoma in the rat was used. Treatment with ICI significantly decreased tumour incidence from 85% to 40% ($p<0.01$) (Fig. 4A). The average tumour number per tumour-bearing animal and the average tumour area were also significantly reduced ($p<0.01$) (Fig. 4B). TE had no significant effects on tumour incidence, tumour number per animal and tumour size as compared to controls ($P<0.01$) (Fig. 4). Dramatic reduction in tumour incidence, tumour number and tumour size was found in the animals who received both ICI and TE ($p<0.01$) (Fig. 4).

Discussion

The Ki-67 labelling index of the epithelium was significantly reduced upon ICI treatment. The reduction in this proliferative marker was accompanied by a significant reduction in epithelial cells. Treatment with ICI induces atrophy of the mammary gland, characterized by a decrease in the size and number of the lobular structure and diminution of secretory activity. Such data indicate the potent antiestrogenic activity of ICI in the mammary gland. This observation gave insight into the novel action of anti-estrogens that may be related to their anti-proliferative and anti-neoplastic activity seen clinically and in animal models.

The Ki-67 labelling index was significantly increased by TE. The potent stimulatory effects of TE was noticed on mammary histomorphology and structure of the female rats. Testosterone enanthate induced a lobuloalveolar type of development of the mammary gland and marked stimulation of the ductal and mainly the lobular structure. In addition, epithelial cell hypertrophy and a marked stimulation of secretory activity were seen, these effects being accompanied by the accumulation of clear and eosinophilic vacuoles in the cytoplasm of the acinar cells. The data are in agreement with previous observations where testosterone caused lobuloalveolar development in both rhesus monkey and rat (27,28) and linked to an increased in breast cancer risk (Reviewd in (21)).

Although DHEA has been shown to exert a potent inhibitory effect on the development of DMBA-induced mammary carcinoma in the rat (20,29), we did not observe any protective effects of testosterone enanthate on DMBA-induced mammary carcinoma. The differences in the anti-tumour activity between DHEA and TE were unclear. Experiments are underway to

determine the differences in anti-tumour activity of DHEA and TE. The observation that TE increased Ki-67 labeling index suggests that *in vivo* TE induced breast epithelial cell proliferation in an autocrine or paracrine fashion. Since androgen receptors were not found in stromal cells of mammary gland (21), the effect of androgens on the stroma, probably through a paracrine action of epithelial cells. The stroma may, in turn, promote mammary epithelial cell proliferation in a reciprocal fashion.

Mammary epithelial cells became terminally differentiated when both ICI and TE were coadministered. Morphological differentiation of epithelial cells in ICI and TE treated glands was quite similar to that observed at the end of pregnancy and during lactation as indicated by increased secretory activity, lipid accumulation and basal nuclei. ICI when given with TE blocked TE-induced Ki-67 expression. Our data provide an explanation for previous observation that combined treatment with fluoxymesterone and tamoxifen have provided survival benefits in advanced breast cancer (30).

Our data demonstrate that Both TE and ICI are required for mammary epithelial cells to express whey acidic protein, α-casein and β-casein. In addition, mammary-derived growth inhibitor MDGI gene expression was greatly induced when both ICI and TE were coadministered. Since WAP, α-casein, β-casein and MDGI genes are preferentially expressed by terminally differentiated mammary epithelial cells (26), the presence of their transcripts provide a good marker for breast differentiation. At the moment, the exact molecular mechanisms responsible for the interaction between TE and ICI in breast epithelial differentiation and anti-proliferation are unknown. Our data suggest that absence of estrogen activity was permissive for induction of milk protein gene and MDGI gene expression and mammary epithelial cell differentiation.

Using the rat DMBA-induced breast carcinoma, we demonstrate that ICI could effectively decrease tumour incidence, the average tumour number per tumour-bearing animal and the average tumour area. TE had no significant effects on tumour incidence, tumour number per animal and tumour size as compared to controls. Dramatic reduction in tumour incidence, tumour number and tumour size was found in the animals who received both ICI and TE suggesting that ICI and testosterone enanthate exerted more potent inhibitory effects than each compound used alone on the development of DMBA-induced rat mammary carcinoma. The observations was compatible with previous report showing that dehydroepiandrosterone and anti-estrogen EM-800 could block the development of DMBA-induced mammary carcinoma in the rat (20). Reduction in tumour incidence induced by DMBA following ICI and TE combined administration may be due to reduction in mammary DNA synthesis and terminal differentiation. Further enhancement in expression of MDGI gene which has a tumour suppressor function (31) may reduce the transformed phenotype of breast cancer cells. Thus we would expect that if the carcinogen is administered during or after ICI and TE treatment, the incidence of mammary tumours would be expected to decrease sharply when compared with incidence of non-treated or single treatment.

In developed countries, reproductive behaviour is determined by both social and economic forces. Thus, for educational, career-related and other reasons, millions of women in these countries are delaying childbearing and having fewer children, in general, than their mother and grandmothers did. Unfortunately, such life decisions will lead to higher rates of breast and ovarian cancer. It is impractical to suggest early pregnancy as a breast cancer prevention strategy. Therefore, a novel approach to breast cancer prevention, at least in certain populations, using physiological signals that encourage terminal differentiation to lower breast cancer risk

was needed. The above described data clearly demonstrate the additive chemopreventive effects of the antiestrogen ICI 182780 and testosterone enanthate on the development of mammary carcinoma as well as differentiative effects of such a combination on mammary epithelial cells. This hormonal regime, when given to women in their teens or early twenties, stimulates mammary differentiation and could potentially reduce the risks for breast cancer. This approach would in fact represent a mimicking of the well-known protective effect of early pregnancy on subsequent breast cancer (5). This strategy is a more physiological approach than long-term administration of tamoxifen, as it seeks to mimic the physiology associated with reproductive behaviour that naturally reduces breast cancer risk. Although our data demonstrate that co-administration of ICI 182780 and testosterone enanthate to rats can differentiate mammary epithelial cells, clinical trials are further needed to clarify the effects of these drugs in human.

Acknowledgements

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Figure Legends

Figure 1. Mammary gland histology in rats treated with vehicles (A), ICI (1 mg/kg/week) (B), TE (1.2 μg/day) (C) or TE plus ICI (D). Note ICI caused marked atrophy of the mammary gland, small atrophic alveoli, lined by atrophic and low cuboidal cells. TE stimulated lobuloalveolar and ductal growth, as well as the secretory activity of acinar cells. TE plus ICI increased lobuloalveolar tissue which consists of groups of alveoli lined by hypertrophic epithelial cells filled with secretory vacuoles similar to that observed in the mammary gland at the end of pregnancy. Haematoxylin-eosine stain was used; magnification. x800.

Figure 2. Effects of treatment with ICI (1mg/kg BW/week) or testosterone enanthate (1.2 μg/day) alone or TE plus ICI on proliferation of mammary epithelial cells. ICI either alone or in combination with TE is very effective in blocking Ki-67 indices of epithelial cells. Bars are different letter are significantly different from one another at $P<0.01$). Data are expressed as the mean ± SEM.

Figure 3. Effects of ICI (1mg/kg BW/week) or testosterone Enanthate (1.2 μg/day) alone or in combination on α-casein, ß-casein, WAP and MDGI gene expression. Rats were treated with ICI, TE and TE plus ICI as described under Materials and Methods. Total RNA derived from mammary gland was subjected to Northern blot. Blots were hybridized with rat α-casein, β-casein, WAP, MDGI and GAPDH cDNAs. For each treatment, three representative samples are shown.

Figure 4. Effects of treatment with ICI (1mg/kg BW/week) or testosterone enanthate (1.2 μg/day) alone or in combination for 150 days on (A) the incidence of DMBA-induced mammary carcinoma, (B) on average tumour number per tumour-bearing animals, and (C) on average tumour size per tumour-bearing rat. Note that ICI treatment caused a significant reduction in tumour incidence, tumour number and tumour size as compared to vehicle and TE treatments. TE plus ICI further decreased the above parameters. Bars in different letter are significantly different from one another at $P<0.01$). Data are expressed as the mean ± SEM.

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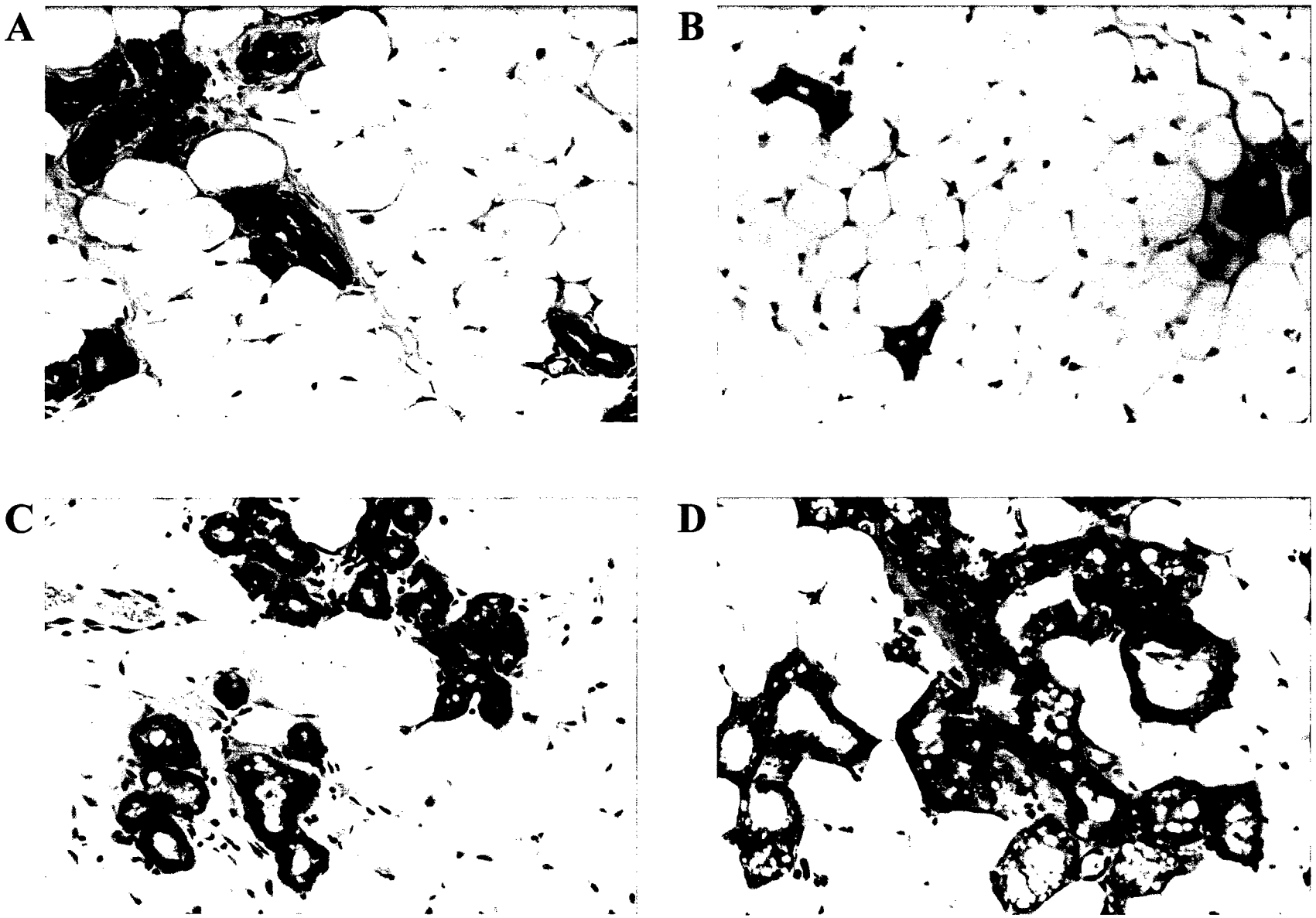


A: Control; B: ICI; C: T; D: ICI + T

FIGURE 1

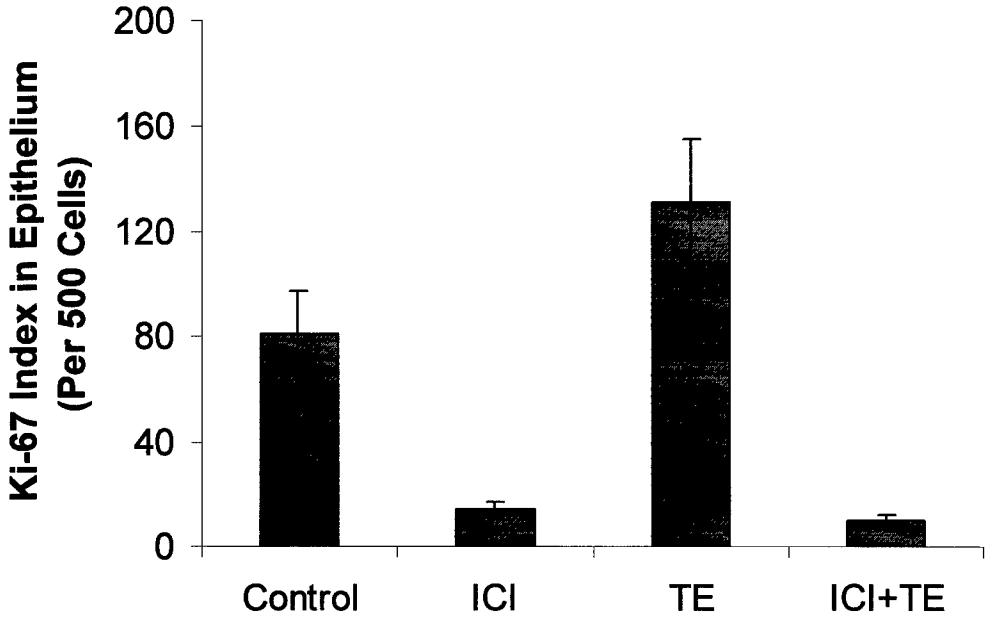

Ki-67 Index in Epithelium
(Per 500 Cells)
200
160
120
80
40
0
Control
ICI
TE
ICI+TE

FIGURE 2

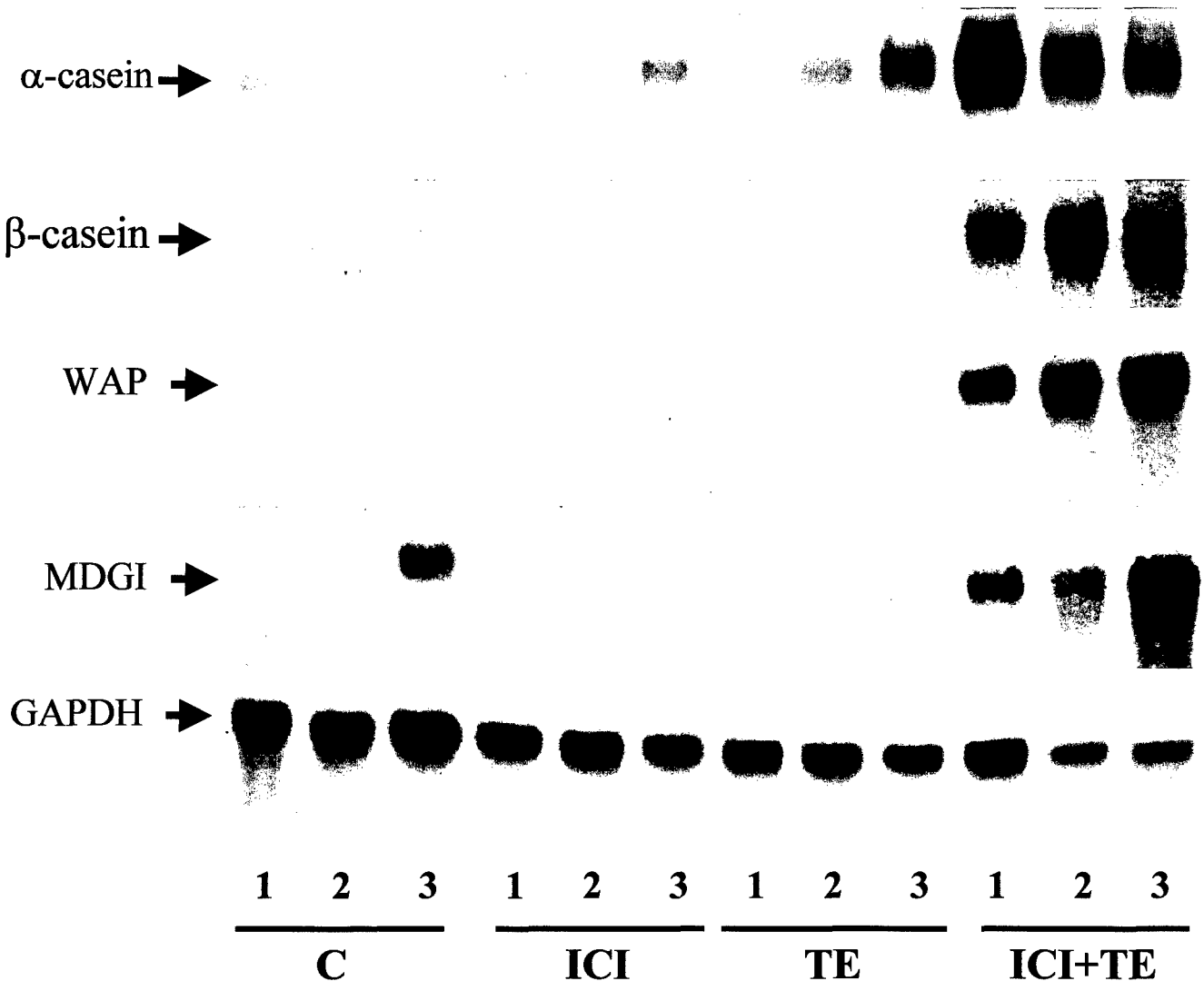
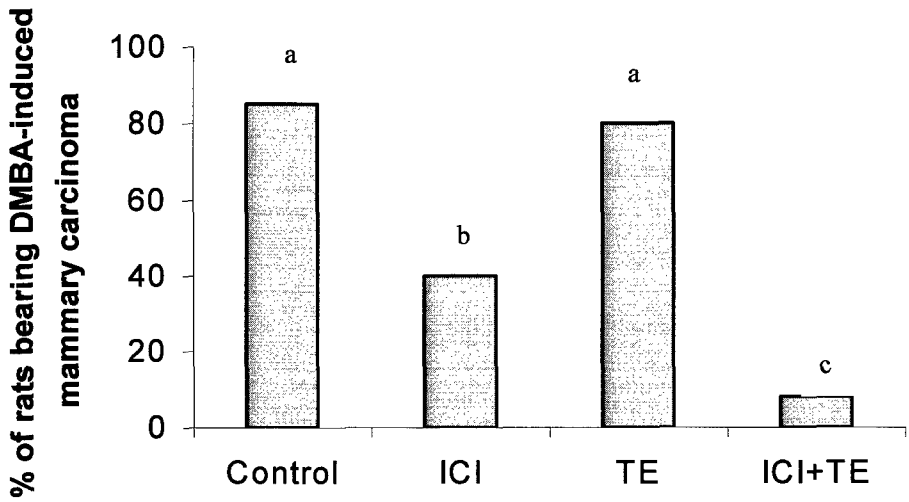
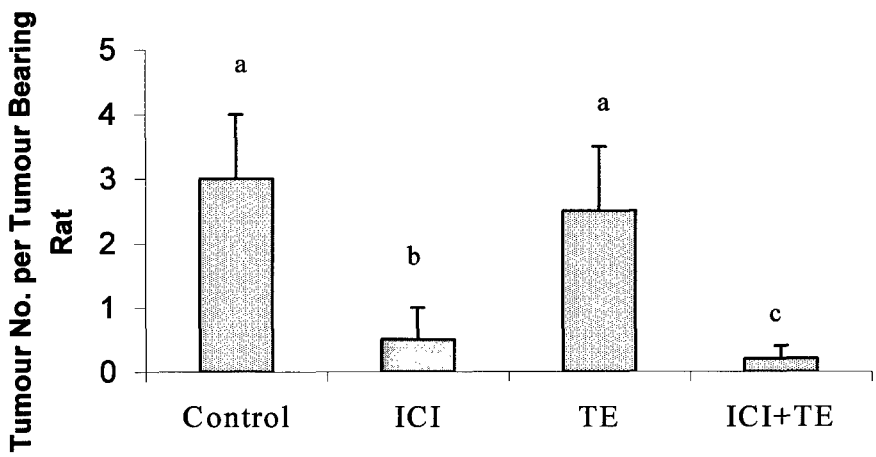


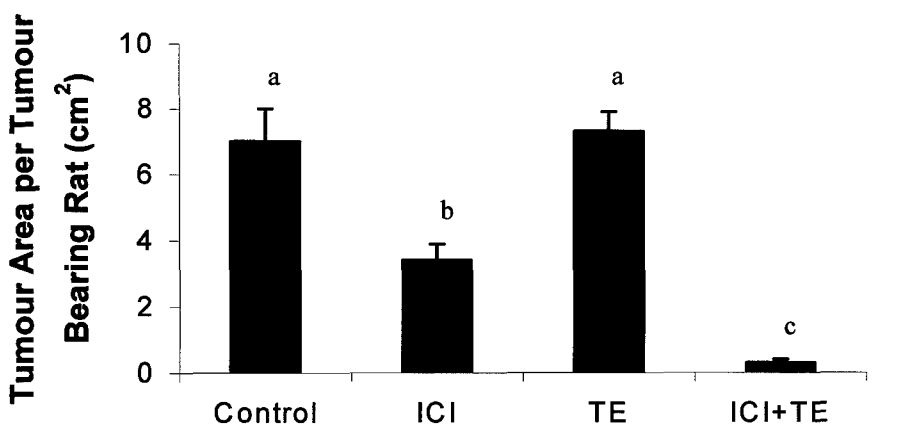
FIGURE 3


% of rats bearing DMBA-induced mammary carcinoma
100
80
60
40
20
0
a
b
a
c
Control
ICI
TE
ICI+TE

A


Tumour No. per Tumour Bearing Rat
5
4
3
2
1
0
a
b
a
c
Control
ICI
TE
ICI+TE

B


Tumour Area per Tumour Bearing Rat (cm2)
10
8
6
4
2
0
a
b
a
c
Control
ICI
TE
ICI+TE

C

Figure 4

Inhibition of Insulin-like Growth Factor Signalling Pathways in Mammary Gland by Pure Anti-estrogen ICI 182780

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Running Title: Inhibition of IGF system by anti-estrogens
Key words: inhibition, IGF, mammary, anti-estrogens

ABSTRACT

The antiestrogens, ICI 182780 (ICI) and tamoxifen, prove to be clinically useful for the treatment of estrogen receptor positive breast tumours. We have assessed the *in vivo* effects of ICI, tamoxifen and estradiol on IGF signalling pathway in the mammary gland of a rat model. ICI significantly decreased the size of the lobular structures, ki-67 labelling index, and insulin-like growth factor binding protein (IGFBP) 2 and -5 gene expression. Treatment of rats with 1, 1.5 and 2 mg ICI/kg BW/week resulted in a 2, 7 and 8-fold increase in IGFBP-3 transcripts. High dose of ICI increased mammary IGF-I gene expression by 2-fold ($p<0.01$). ICI decreased IGF-IR and its basal phosphorylation to approximately 30% of the control mammary gland. IRS-1, IRS-2 and c-Raf-1 levels in the ICI-treated mammary glands were about 30%, 15% and 40% of controls, respectively. Basal phosphorylation of IRS-1 and PI-3 kinase p85, but not IRS-2, were barely detectable following ICI treatment. Despite significant reduction in phospho IGF-IR, IRS-1 and IRS-2, phospho p42/p44 MAPK levels were slightly decreased. Degradation of phospho p42/p44 proteins were observed. Tamoxifen-induced growth inhibition was associated with slight stimulation of IGFBP-3 gene expression and reduction in IRS-2 levels. Basal phosphorylation of IGF-IR, IRS-1 and PI-3 kinase p85 was decreased. Estradiol-induced epithelial cell proliferation was associated with inhibition of IGFBP-3 gene expression, stimulation of IGFBP-2 gene expression, increase in IGF-IR, IRS-1, IRS-2, and c-Raf-1 levels. Despite basal phosphorylation of IGF-IR, IRS-1, IRS-2 and PI-3 kinase p85 was significantly increased by estradiol, basal phospho MAPK p44/42 was significantly reduced ($p<0.01$). Because the IGF system plays an important role in breast epithelial cells proliferation, metastasis and apoptosis, the described activities of antiestrogens may contribute to its anti-proliferative properties seen clinically and in animal models.

INTRODUCTION

Epidemiological studies have shown a link between IGF-I and risk of breast cancer. Among premenopausal women less than 50 years of age, there was a 4.5-fold relative risk of breast cancer in the highest quartile of plasma IGF-I compared with the lowest quartile (1). IGFs are mitogenic and anti-apoptotic agents for breast epithelial cells *in vitro* (2,3). IGFs exert their effects through IGF-I receptor (IGF-IR). Target disruption of the IGF-IR resulted in cell refractoriness to viral and cellular oncogenes, increased the probability of apoptosis and inhibited neoplastic proliferation (4-6). *In vivo* studies demonstrated that terminal end bud of mammary gland was impaired in the absence of IGF-I (7-9). IGF-I synergized along with estrogens to stimulate ductal morphogenesis (7). Treatment of mice with estradiol had no effect on mammary development in IGF-I null mice. Thus IGF-I is a factor that permits estrogens to act.

IGF action was modulated by IGF binding proteins (IGFBPs) which are potential mediators of apoptosis (10). Six high affinity IGFBPs have been described (10-12). There is clear evidence that they modulate activity of IGFs. IGFBP-3 was negatively related to breast cancer risk (1). We and others have shown that IGFBP-3 also had growth inhibitory activity that was independent of its IGF binding properties (13-15).

IGF binding activates the intrinsic tyrosine kinase activity, induces IGF-Receptor (IGF-1R) autophosphorylation. Phosphorylated IGF-IR phosphorylates IRS-1, IRS-2, and Shc and activates the signal transduction pathways, such as phosphatidyl inositol-3 kinase (PI-3K) and Ras/Raf/mitogen-activated protein kinase (MAPK) (16). The first signalling cascade involves

activation of PI-3 kinase and subsequent formation of phosphatidyl inositol-3 phosphate, which can serve as a signal for cell growth. The Ras/Raf/MAPK pathway involves the extracellular signal related kinases ERK1 and ERK2 (MAPKs). Phosphorylation of IRS-1 by the IGF-IR results in the formation of an IRS-1-Grb2-Sos complex which activates Ras. Activated Ras p21 binds Raf-1 and activates Raf-1, which results in the phosphorylation and activation of ERKs, which in turn transmit a signal to the nucleus (17). Ras/Raf/MAPK and PI-3K have been identified as playing important roles in IGF-IR-induced cellular proliferation and the inhibition of apoptosis. The Ras/Raf/MAP kinase pathway was thought to primarily mediate the cell proliferative response to growth factors such as IGFs, whereas the PI-3 kinase pathway, which activates Akt was primarily implicated in mediating anti-apoptotic effects of IGFs (18).

In women, a high proportion of primary breast cancers contain the estrogen receptor (ER) and require estrogen or estrogenic activities for tumour growth. Current therapies have been directed toward interruption of estrogen by oophorectomy or the use of antiestrogens (19,20). The antiestrogen drug tamoxifen improved the survival of women with breast cancer, and has proven to be clinically useful for the treatment of metastatic ER-positive tumours (21,22). It has been proposed that the inhibitory effect of these compounds on IGF-I expression contributes to their antiproliferative activity (23,24,25). Unfortunately, the overwhelming majority of tumours progressed to a phenotype characterized by resistance to tamoxifen, thus restricting clinical use of this drug (19). The pure antiestrogen ICI 182780 (ICI) (22) exhibited effects on some patients following disease progression on tamoxifen (20). ICI has recently been shown to act as a growth inhibitor even in the complete absence of estrogen stimuli (14,26), and to actively regulate gene expression in a direction opposite to that of estrogens (14). We and others have shown that ICI had a

stronger antineoplastic activity than tamoxifen and also lack uterine side effects of tamoxifen (27).

Here we reported that treatment of rats with a pure antiestrogen ICI 182780 increased IGF-I and IGFBP-3 gene expression. ICI and tamoxifen inhibited expression and basal phosphorylation of several key proteins involved in IGF signal cascades. Since the IGF system plays an important role in breast cancer cell proliferation, metastasis and apoptosis, the described activities of tamoxifen and ICI may contribute to their anti-proliferative and anti-neoplastic activity seen clinically and in animal models.

Materials and Methods

Animal experiments were approved by Local Animal Care Committee. Ovary intact Sprague-Dawley female rat, 60 days old at the beginning of the experiments, were obtained from Charles River, Quebec. To investigate the effects of ICI 182780 on expression of IGF system in the mammary gland, rats were weekly injected with 1 mg, 1.5 mg, and 2 mg/kg BW ICI 182780 (AstraZeneca, Pharmaceuticals) in castor oil for 3 weeks. Control rats received only castor oil. For estradiol studies, groups of rats were implanted with 0.5 cm silastic tubes (0.04 in. ID, Dow Corning, Michigan) containing 17 β-oestradiol (Sigma) on the back of their neck. Control rats were experienced the same surgical implantation with empty silastic tubes. Based on previous published work (28), the released rate of 17 β-oestradiol from silastic implants was documented to be 2.4 μg/cm/day. To examine the effects of tamoxifen on expression of genes in the IGF system, rats were implanted with either 4 cm silastic tubes (0.12 in. ID, Dow corning, Michigan) containing tamoxifen (Sigma) on the back of their neck. The released rate was approximately 25 μg/cm per day. Animals

were sacrificed by carbon dioxide at the end of the experiment. The mammary tissue was excised, trimmed and frozen in liquid nitrogen and stored at -70 ^{0}C for RNA extraction. Part of the mammary tissue was fixed in 10% buffered formalin for histochemical studies.

Immunohistochemistry and histology: Fixed mammary tissue was routinely processed in a tissue processor and embedded in paraffin. Sections of 5 μm were cut and stained with haematoxylin-eosin. Examination of the slides was performed by light microscopy. The ImmunoCruz Staining System was used for immunohistochemical study. Briefly, the slides were deparaffinized, rehydrated in water and incubated with 3% H_2O_2 for 20 min to block endogenous peroxidase activity. To examine expression of Ki-67, antigens were retrieved by heating the slides in citrate buffer (pH 6) for 5 min. After preincubation with normal serum for 20 min at room temperature, the primary antibody was applied (2 μg/ml) and incubated overnight at 4 ^{0}C. The section was then incubated with the appropriate biotinylated secondary antibody at 1:500 dilution followed by peroxidase-conjugated streptavidin complex according to the manufacturer's instruction and DAB. The section was then counterstained with haematoxylin. Between each change of incubation the sections were rinsed 3 times in phosphate buffer saline for 5 min each. To evaluate the Ki-67 labelling index, 500 epithelial cells were counted for each group in randomly chosen fields at an x 400 magnification. The Ki-67 labelling index was expressed as the number of clearly labelled Ki-67 reactive nuclei in 500 cells counted. Significance difference was determined by Student t-test.

Western Blotting. To determine the changes in the expression of PI-3K p85, c-Raf-1, IGF-IR, IRS-1, IRS-2 and phospho p44/42 MAPK, mammary tissue was homogenized in lysis buffer (1 mM $CaCl_2$, 1 mM $MgCl_2$, 1% NP-40, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μM PMSF, and 100 μM

$NaVO_4$). Proteins were subjected to Western blot analysis as described (29). Blots were incubated with indicated antibody and horseradish peroxidase-conjugated donkey anti-mouse or anti-rabbit secondary antibody (1:7500). Blots were visualized with a chemiluminescent detection system as described by the manufacturer (ECL, Amersham). Rabbit anti- PI-3K p85, rabbit anti-c-Raf-1, mouse anti-α tubulin and rabbit anti-IGF-IR antibodies were purchased from Santa Cruz. Rabbit anti-IRS-1 and rabbit anti-IRS-2 antibodies were obtained from Upstate Biotechnology. Mouse anti-phospho specific MAPK antibody was from New England BioLabs.

Basal phosphorylation of IGF-IR, IRS-1 and IRS-2 were determined by immunoprecipitation of total cellular lysates using anti-IGF-IR, IRS-1 and IRS-2, respectively. Briefly, 500 μg of total cellular proteins were incubated with 1 or 2 μg of primary antibody for 1 hour at 4 ^{0}C. Immunoprecipitates were collected using protein A/G Plus-Agarose. After washing 4 times with lysate buffer, the pellets were resuspended in electrophoresis sample buffer and boiled for 3 minutes. Immunoprecipitated proteins were analyzed by Western blotting using anti-phosphotyrosine antibodies (Upstate Biotechnology).

Northern Blotting. Total RNA was isolated from mammary tissue and Northern blotting was performed as described (24). Blots were hybridized with IGF-I (30) and IGFBP (1-5) (11) cDNAs. To control for equal RNA loading, blots were rehybridized with GAPDH cDNA (ATCC). Quantitative analysis of gene expression was accomplished by scanning autoradiograms and densitometry. For each lane, the sum of the density of bands corresponding to transcripts hybridizing with the probe under study was calculated, and normalized with the amount of RNA loaded.

RESULTS

Control mammary gland had a sparse cluster of epithelial tubules surrounded by a small amount of connective tissue which was in turn embedded in a large fat pad. The epithelial ducts had a small lumen, lined by cuboidal cells with dark stained nuclei (Fig. 1A). Treatment of ovary intact animals with ICI 182780 resulted in a marked atrophy of the mammary gland. The ICI-induced pattern was characterized by a decreased size of the lobular structures, which consisted of small atrophic alveoli, lined by atrophic and low cuboidal cells. The acinar epithelial cells were apparently inactive, with diminished quantity of cytoplasm (Fig. 1B). Similar histological observations were also seen when rats were treated with tamoxifen (Fig. 1D). Estradiol extensively stimulated lobuloalveolar, ductal and epithelial cell growth (Fig. 1C)

We determined the effect of antiestrogens and estradiol on Ki-67 expression in mammary tissue. Figure 2 shows the results of an experiment where mammary tissues were collected from rats treated with vehicle, ICI, 17-β estradiol and tamoxifen for immunohistochemical analysis of Ki-67 expression. Estradiol significantly increased ($P<0.01$) while ICI and tamoxifen significantly decreased ($P<0.01$) the number of epithelial cells expressing Ki-67 protein as compared to controls.

Since IGF-I is potent mitogen for normal and breast cancer cells and IGFBPs modulate activity of IGF-I (31), the effects of ICI on mammary IGF-I and IGFBP gene expression were examined. As shown in figure 3, ICI significantly increased IGF-I mRNA by 2-fold ($p<0.05$) while estradiol and tamoxifen had no significant effect on IGF-I gene expression. ICI also induced IGFBP-3 mRNA accumulation in a dose-dependent manner (Fig. 4A) while IGFBP-2 and 5 gene expression were

significantly inhibited ($p<0.01$) (Fig. 5). Estradiol significantly inhibited IGFBP-3 ($P<0.01$) (Fig. 4A) and stimulated IGFBP-2 gene expression (Fig. 5). Tamoxifen mildly enhanced IGFBP-3 gene expression (Fig. 4A).

Since over-expression of IGF-IR has been reported in a variety of tumours (32) and *in vivo* over-expression of this receptor protected cells from apoptosis (33,34), the effects of estradiol and antiestrogens on IGF-IR and its basal phosphorylation were examined. ICI treatment, but not tamoxifen, significantly reduced IGF-IR levels compared to controls ($p<0.01$) (Fig. 6A). Estradiol significantly increased IGF-IR levels ($P<0.01$). As shown in figure 6C, basal phosphorylation of IGF-IR was significantly low in ICI- and tamoxifen-treated mammary glands ($p<0.05$). Despite high levels of IGF-IR protein, the basal phosphorylation of IGF-IR in estradiol-treated mammary tissue was slightly decreased.

To investigate whether antiestrogens and estradiol affected IRS-1 and IRS-2 levels and their basal phosphorylation, Western blot analysis was performed. As shown in figure 7, ICI significantly reduced both IRS-2 but IRS-1 levels ($p<0.01$). Tamoxifen selectively reduced IRS-2 but not IRS-1. Basal phosphorylation of IRS-1 was significantly reduced following ICI and tamoxifen treatments ($p<0.01$). Basal IRS-2 phosphorylation in ICI- and tamoxifen-treated mammary glands was about 35% and 80% of that seen in control mammary glands, respectively (Fig. 8). Estradiol significantly increased both IRS-1 and IRS-2 as well as their basal phosphorylation ($p<0.01$).

Since the phosphorylated IGF-IR phosphorylates IRS-1 and IRS-2, and activates the signal Ras/Raf/mitogen-activated protein kinase (MAPK) pathways, the levels of c-Raf-1 and phospho

MAPK were determined (18). As shown in figure 9, c-Raf-1 protein was decreased by 50% by ICI ($p<0.01$). Estradiol increased c-raf-1 by 2-fold ($P<0.01$). Tamoxifen slightly increased c-Raf-1. Despite significant reduction in levels of IGF-IR, IRS-1, IRS-2 and c-Raf-1, the levels of phospho p44/42 MAPK was slightly decreased by ICI and tamoxifen-treatments. Degradation of phospho MAPK was observed in all ICI-treated samples (Fig. 10). Basal phosphorylation of MAPK p44/42 was significantly low in estradiol-treated mammary tissue as compared to vehicle and antiestrogen-treated mammary glands ($P<0.01$) (Fig. 10).

Since PI-3K activity is very important for IGF-induced mitogenesis and IRS-1 and IRS-2 proteins mediate activation of PI-3K by IGF-IR which, when phosphorylated recruits subunit p85 of PI-3K, leading to activation of its enzymatic activity (35,36), the levels of PI-3K p85 and its phosphorylation were investigated. Figure 11 shows that levels of PI-3 kinase p85 did not change as a result of ICI and tamoxifen treatments. However, the basal phosphorylation of PI-3 kinase p85 was undectable following ICI- and tamoxifen-treatments. Estradiol, on the other hand, significantly decreased unphosphorylated PI-3 kinase p85 and significantly increased basal phosphorylation of PI-3 kinase p85 ($p<0.01$).

Discussion

The pure antiestrogen ICI 182780 and tamoxifen prove to be clinically useful for the treatment of estrogen receptor positive breast tumours. In this study, we report that treatment with either ICI or tamoxifen induced atrophy of the mammary gland, characterized by a decrease in the size and number of the lobular structure and diminution of secretory activity. The Ki-67 labelling index of the

epithelium was significantly reduced upon ICI and tamoxifen treatments. The reduction in this proliferative marker was accompanied by a significant reduction in breast epithelial cells.

Our *in vivo* studies revealed that ICI increased IGF-I gene expression and inhibited IGFBP-2 and IGFBP-5. ICI-inhibited breast epithelial cell growth was associated with upregulation of IGFBP-3 gene expression, decrease in IGF-IR and its basal phosphorylation, and inhibition of IRS-1, IRS-2 and c-Raf-1 expression. Phosphorylation of IRS-1, but not IRS-2, protein was undetectable following ICI treatment. Phospho p42/p44 MAPK levels were slightly decreased. Degradation of phospho p42/p44 proteins were observed. PI-3 kinase p85 levels were not affected by ICI treatment while basal phospho PI-3K p85 was inhibited. Tamoxifen had a mild effect on IGFBP-3 gene expression. Inhibition of breast epithelial cell proliferation was associated with reduction in basal phosphorylation of IGF-IR, IRS-1 and PI-3 kinase p85. Induction of breast epithelial cell proliferation by estradiol was associated with increase in IGF-IR, c-Raf-1, IRS-1, IRS-2 and reduction in IGFBP-3. More importantly, estradiol increased basal phosphorylation of IRS-1, IRS-2 and PI-3K p85 and decreased MAPK phosphorylation. Such data indicate the potent anti-estrogenic activity of ICI and tamoxifen (lesser extent) in the mammary gland. These observations gave insight into the action of anti-estrogens that may be related to their anti-proliferative and anti-neoplastic activity seen clinically and in animal models.

Since stromal, but not epithelial, cells express IGF-I, the observed increase in IGF-I mRNA following ICI treatment may be due to high stromal/epithelial cell ratio. The magnitude of ICI increased IGF-I gene expression is far lesser than its ability to induce IGFBP-3 gene expression. The antiproliferation activity of antiestrogens on breast epithelial cells are well correlated with their

effects on IGFBP-3 expression, assuming a growth inhibitory action of this protein: the weak inhibitory effect of tamoxifen is associated with mild stimulation of mammary IGFBP-3 expression, while ICI 182780-induced growth inhibition is associated with upregulation of mammary IGFBP-3 expression (14). In view of these results, we observed that *in vivo* tamoxifen is less potent than ICI 182780 in upregulating IGFBP-3 mRNA abundance in rat mammary gland and that maximum stimulation was only to ~125% of control values, as compared to the 8-fold stimulation seen with ICI. Our data demonstrate a consistent relationship between the effects of antiestrogens on proliferation and on IGFBP-3 expression. ICI action *in vivo* described here may involve not only IGFBP-3 from the epithelial cell autocrine loop but also a paracrine source of IGFBP-3 from stromal fibroblasts. Given the importance of IGF-I in proliferation, co-operative enhancement of estrogen signalling (37), and inhibition of programmed cell death (18), reduction in IGF-I bioactivity by upregulation of IGFBP-3 will undoubtedly have consequences on the growth of tumour.

The functional significance of IGFBP-2 and IGFBP-5 within the mammary gland and breast epithelial growth is unknown. *In vitro* both inhibition and potentiation of IGF activity by IGFBP-2 and -5 have been reported (reviewed in (31)). Our data demonstrate that IGFBP-2 expression is stimulated by estrogen and inhibited by ICI and positively correlates with breast epithelial cell proliferation. It has been reported that IGFBP-2 levels were elevated in serum from various cancer patients (38-41) and over-expression of IGFBP-2 resulted in increased tumorigenic potential of adrenocortical cells (42). It is possible that increased IGFBP-2 may potentiate the response to IGF-I by breast epithelial cells. In the present study, IGFBP-5 expression is inversely correlated with ICI-induced growth inhibition which is different from previously reported (43) where IGFBP-5 expression was greatly induced during mammary involution.

The important role of IGF-IR in autocrine/paracrine activation of the IGF pathway in tumours (32), anti-apoptosis (5,6) and sensitivity to chemotherapy drugs (44) was well documented. Down regulation of IGF-IR by 30 to 80% was sufficient to reverse the transformation of human cervical cancer cells (45). By the same token, *in vivo* down-regulation of IGF-IR and its basal phosphorylation by ICI and tamoxifen (lesser extent) would expect to interfere the IGF signal cascade leading to inhibition of cellular proliferation and enhancement of apoptosis. Experiments are underway to examine if antiestrogens also induce apoptosis in mammary gland. Interruption of IGF-IR signalling may also make tumour cells more sensitive to apoptotic induced agents. Our findings emphasized the potential usefulness of ICI in conjunction with chemotherapy drugs in treatment of breast cancer.

Although activation of MAPK is required for IGF-I-induced proliferation in breast cancer cells (44), we observed high levels of phospho MAPK following tamoxifen- and ICI-induced growth arrest. Furthermore, estradiol-induced proliferation was associated with reduction in basal phosphorylation of MAPK. Although the nuclear translocation of MAPK has not been determined in our present study, it is possible that the duration of MAPK activation and nuclear translocation of the enzyme induced by ICI and estradiol may give difference in the biological actions of ICI and estradiol on breast epithelial cell proliferation. The substrate of MAPK include transcription factors and other kinases (46-48). Phosphorylation of these transcription factors by MAPK may lead to induction of expression of new genes which are responsible for anti-proliferative effect. This hypothesis is supported by the observations that in PC12 cells, nerve growth factor induced growth arrest was associated with sustained activation and nuclear translocation of MAPK while insulin or EGF induced proliferation was associated with transient activation of MAPK without pronounced nuclear

translocation of the enzyme (48,49). Experiments are under way to determine the duration of MAPK activation and nuclear translocation of MAPK following estradiol and ICI treatment.

It has been reported that IGF-I can rescue breast cancer cells from doxorubicin-induced apoptosis and the process requires PI-3 kinase (44). Recent work into IGF-I anti-apoptosis signalling has demonstrated the importance of PI-3 kinase and its downstream substrate Akt (50,51). Furthermore, a direct link between PI-3 kinase and apoptosis-regulating protein Bcl family of proteins has established through Akt phosphorylation of Bad (52,53). Thus the ability of both tamoxifen and ICI to inactivate PI-3 kinase by inhibition of PI-3K p85 phosphorylation may be important for antiestrogen-induced apoptosis. Experiments are under way to determine whether ICI and tamoxifen also induce breast epithelial apoptosis *in vivo*.

It has been reported that IGF-I synergies along with estrogens to stimulate ductal morphogenesis (7) and treatment of mice with estradiol has no effect on mammary development in IGF-I null mice (7). This observation may be due to the enhancement of IGF on the ligand-induced transcriptional activity of estrogen receptor (54). The inhibition of mammary epithelial growth by ICI and tamoxifen observed here may be due to the interference of a possible cross-talk between the estrogen and IGF signalling pathways.

A recent clinical trial (20) suggests that ICI 182780 can induce clinical responses even in breast cancers resistant to tamoxifen, an antiestrogen commonly used clinically. In addition, a recent *in vivo* study demonstrated that tamoxifen is a less potent inhibitor of *in vivo* MCF-7 cell proliferation than ICI 182780 (55). In our present study, we observed that ICI and tamoxifen inhibited IRS-2 and

basal phosphorylation of IGF-IR, IRS-1 and PI-3 p85. In addition, ICI also decreased IGF-IR, c-Raf-1 and IRS-1 expression and strongly enhanced IGFBP-3 gene expression while tamoxifen had very little or no effects on these proteins. These observations may account for the differences in anti-proliferative activity between tamoxifen and ICI seen clinically and in animal models.

In summary, the role of IGF-I in growth, apoptosis, metastasis of breast cancer cells has been established. Inhibition of IGF-I or its actions may provide one of the strategies for treatment of breast cancer. The concept of pharmacological measures to reduce risk is now well established with an on-going large breast prevention trial evaluating the IGF-targeting approach (1). Our data provide evidence for a previously unrecognized aspect of antiestrogen action: the antiproliferative effect of ICI and tamoxifen is related at least in part by inhibition of expression of several proteins involved in the IGF signal pathway and their basal phosphorylation. The observed inhibitory action of ICI likely involves the direct growth inhibitory signal transduction pathway (13,56) as well as reduction of bioavailability of IGFs for cell surface receptor binding. This implies that ICI 182780 does not merely act as a competitive antagonist to block estrogen action, but rather actively regulates genes involved in the IGF signal pathways. These mechanisms may be relevant to the *in vivo* actions of antiestrogens. Our data also raise the possibility that ICI can be used as a single agent or in adjunction with chemotherapy drugs in treatment of patients with breast cancer.

Figure Legends

Figure 1. Mammary gland histology in rats treated with vehicles (A), ICI (2 mg/kg BW/week) (B), tamoxifen (100 μg/day) (C) or estradiol (1.2 μg/day) (D). Note ICI and tamoxifen caused marked atrophy of the mammary gland, small atrophic alveoli, lined by atrophic and low cuboidal cells. Estradiol stimulated lobuloalveolar and ductal growth, as well as the secretory activity of acinar cells. Haematoxylin-eosine stain was used; magnification. x400.

Figure 2. Effects of treatment with vehicles (C), ICI (2 mg/kg BW/week), tamoxifen (100 μg/day) or estradiol (1.2 μg/day) on proliferation of mammary epithelial cells. ICI and tamoxifen were very effective in blocking Ki-67 index of epithelial cells while estradiol stimulated it. Bars are different letter are significantly different from one another at ($p<0.01$). Data are expressed as the mean ± SEM.

Figure 3. Effects of ICI 182780 (ICI), tamoxifen and estradiol on mammary gland IGF-I gene expression. Rats were treated with indicated concentrations of estradiol (1.2 μg/day), tamoxifen (100 μg/day) and ICI (2 mg/kg BW per week). Total RNA derived from mammary gland was subjected to Northern blotting. Blots were hybridized with GAPDH (**A**) and rat IGF-I (**B**). Densitometric scanning of the IGF-I bands are shown in (**C**). Bars with different letters are significantly different from one another at ($p<0.01$). ICI significantly increased IGF-I gene expression while estradiol slightly inhibited it.

Figure 4. Effects of ICI 182780 on mammary gland IGFBP-3 expression. Rats were treated with indicated concentrations of ICI. Total RNA derived from mammary gland was subjected to Northern blot analysis. Blots were hybridized with rat IGFBP-3 (**A**) and GAPDH (**B**) cDNAs. Densitometric scanning of the IGFBP-3 band is shown in (**C**). Bars with different letters are significantly different from one another at ($p<0.01$). ICI induced IGFBP-3 gene expression in a dose dependent fashion.

Figure 5. Effects of ICI 182780 on mammary gland IGFBP-2, -4 and -5 expression. Rats were treated with indicated concentrations of estradiol (1.2 μg/day), tamoxifen (100 μg/day) and ICI (2 mg/kg BW per week). Total RNA derived from mammary gland was subjected to Northern blotting. Blots were hybridized with rat IGFBP-2, -4, -5 and GAPDH DNAs. ICI significantly inhibited IGFBP-2 and -5 gene expression ($p<0.01$).

Figure 6. Effects of ICI, tamoxifen and estradiol on mammary gland IGF-I receptor and its phosphorylation form. Rats were treated with indicated concentrations of estradiol (1.2 μg/day), tamoxifen (100 μg/day) and ICI (2 mg/kg BW per week). Tissue lysates from mammary tissue were analyzed by Western blotting. Blots were incubated with anti-IGF-IR (**A**) and α-tubulin (**B**) antibodies. To determine IGF-IR phosphorylation, tissue lysates were immunoprecipitated with anti-IGF-IR antibody. Following SDS-PAGE, the levels of phosphorylated IGF-IR were detected by immunoblotting using antiphosphotyrosine antibody 4G10 (**C**). Densitometric scanning of the IGF-IR and its tyrosine phosphorylated IGF-IR is shown in (**D**). Bars with different letters are significantly different from one another at ($p<0.01$). ICI significantly decreased both IGF-IR and basal IGF-IR phosphorylation while estradiol increased IGF-IR.

Figure 7. Effects of ICI, tamoxifen and estradiol on IRS-1 and IRS-2 levels in the mammary

gland. Rats were treated with indicated concentrations of estradiol (1.2 μg/day), tamoxifen (100 μg/day) and ICI (2 mg/kg BW per week). Tissue lysates from mammary tissue were analyzed by Western blotting. Blots were incubated with anti-IRS-1 (**A**), IRS-2 (**B**) and α-tubulin (**C**) antibodies. Densitometric scanning of the IRS-1 and IRS-2 is shown in (**D**). Bars with different letters are significantly different from one another. IRS-1 and IRS-2 levels were significantly decreased ($P<0.01$) by ICI.

Figure 8. Effects of ICI, tamoxifen and estradiol on phospho IRS-1 and IRS-2 levels in the mammary gland. Rats were treated with indicated concentrations of estradiol (1.2 μg/day), tamoxifen (100 μg/day) and ICI (2 mg/kg BW per week). Tissue lysates were immunoprecipitated with anti-IRS-1 (**A**), IRS-2 (**B**). Following SDS-PAGE, the levels of phosphorylated IRS-1 and IRS-2 were detected by immunoblotting using antiphosphotyrosine antibody 4G10 (**C**). Densitometric scanning of tyrosine phosphorylated IRS-1 and IRS-2 is shown in (**D**). Bars with different letters are significantly different from one another at ($p<0.01$). Basal IRS-1 phosphorylation was undetectable in ICI-treated mammary gland.

Figure 9. Effects of ICI, tamoxifen and estradiol on c-Raf 1 in the mammary gland. Rats were treated with indicated concentrations of estradiol (1.2 μg/day), tamoxifen (100 μg/day) and ICI (2 mg/kg BW per week). Tissue lysates from mammary tissue were analyzed by Western blotting. Blots were incubated with anti-α-tubulin (**A**) and anti-c-Raf 1 (**B**) antibodies. Densitometric scanning of the c-raf-1 is shown in (**C**). Bars with different letters are significantly different from one another at ($p<0.01$). Estradiol significantly increases while ICI decreases c-Raf-1 levels.

Figure 10. Effects of ICI, tamoxifen and estradiol on phospho p42/44 MAPK levels in the

mammary gland. Rats were treated with indicated concentrations of estradiol (1.2 μg/day), tamoxifen (100 μg/day) and ICI (2 mg/kg BW per week). Tissue lysates from mammary tissue were analyzed by Western blotting. Blots were incubated with phospho-specific p44/42 MAPK (**A**) and anti-α-tubulin (**B**) antibodies. Densitometric scanning of the phospho p44 and p42 is shown in (**C**). Bars are different letter are significantly different from one another at ($p<0.01$). ICI slightly decreased intact phospho p44/42 MAPK by enhancing p44/42 MAPK degradation while estradiol decreased phospho p44/42 MAPK levels.

Figure 11. Effects of ICI, tamoxifen and estradiol on PI-3 kinase p85 in the mammary gland. Rats were treated with indicated concentrations of estradiol (1.2 μg/day), tamoxifen (100 μg/day) and ICI (2 mg/kg BW per week). Tissue lysates from mammary tissue were analyzed by Western blotting. Blots were incubated with anti-α-tubulin (**A**) and anti-PI-3 kinase p85 (**B**) antibodies. Bars with different letters are significantly different from one another at ($p<0.05$) Estradiol significantly induces PI-3 kinase p85 phosphorylation. ICI reduced basal PI-3 p85 phosphorylation without affecting PI-3 kinase p85 levels.

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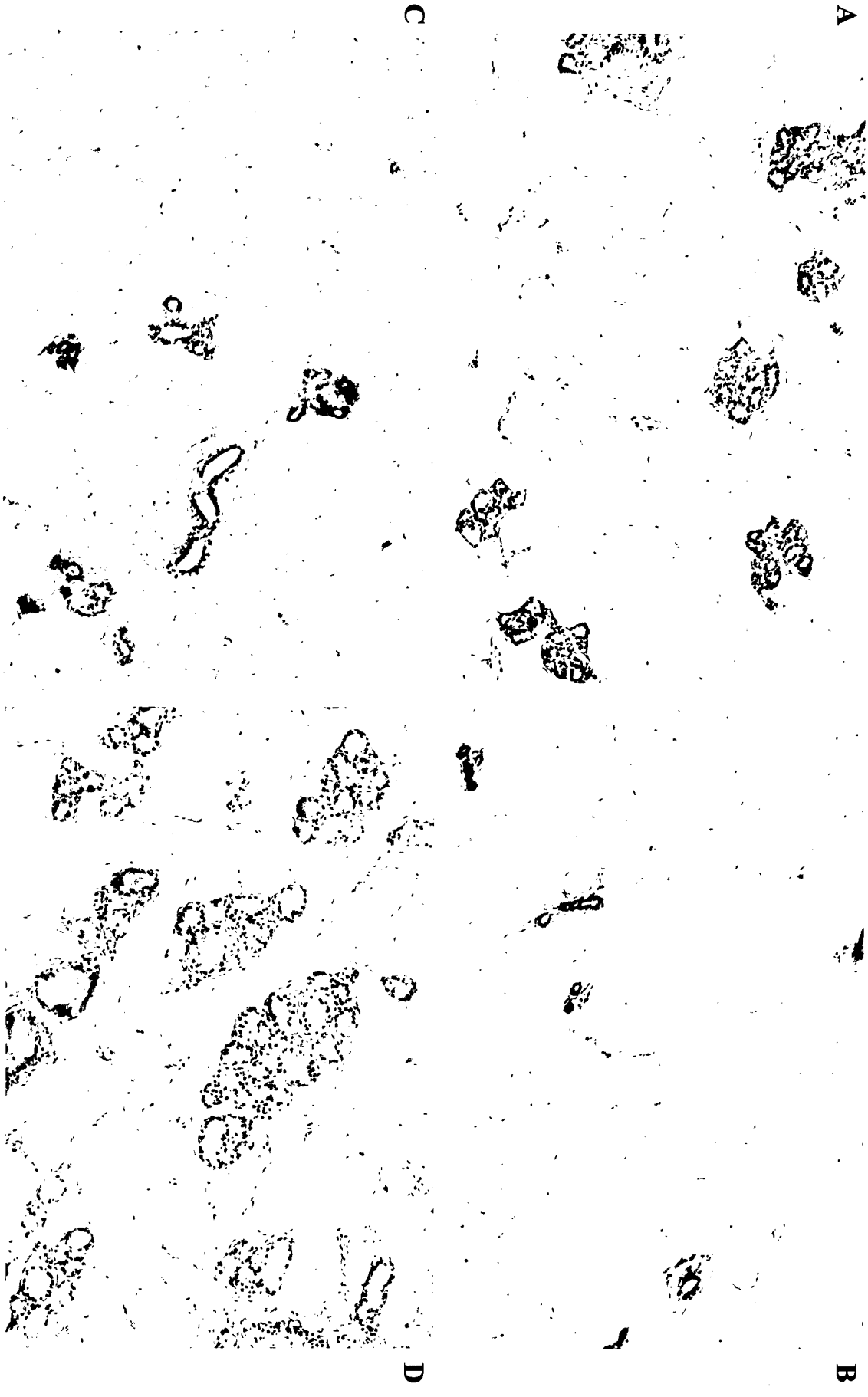


FIGURE 1

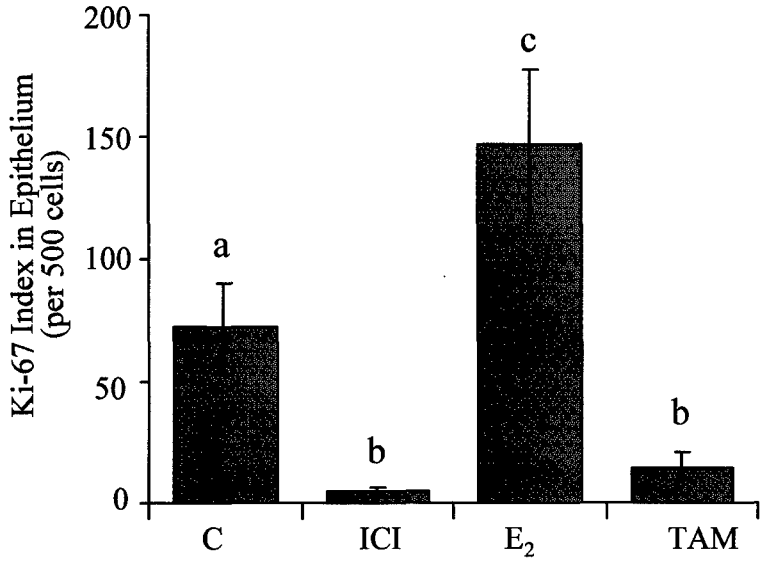
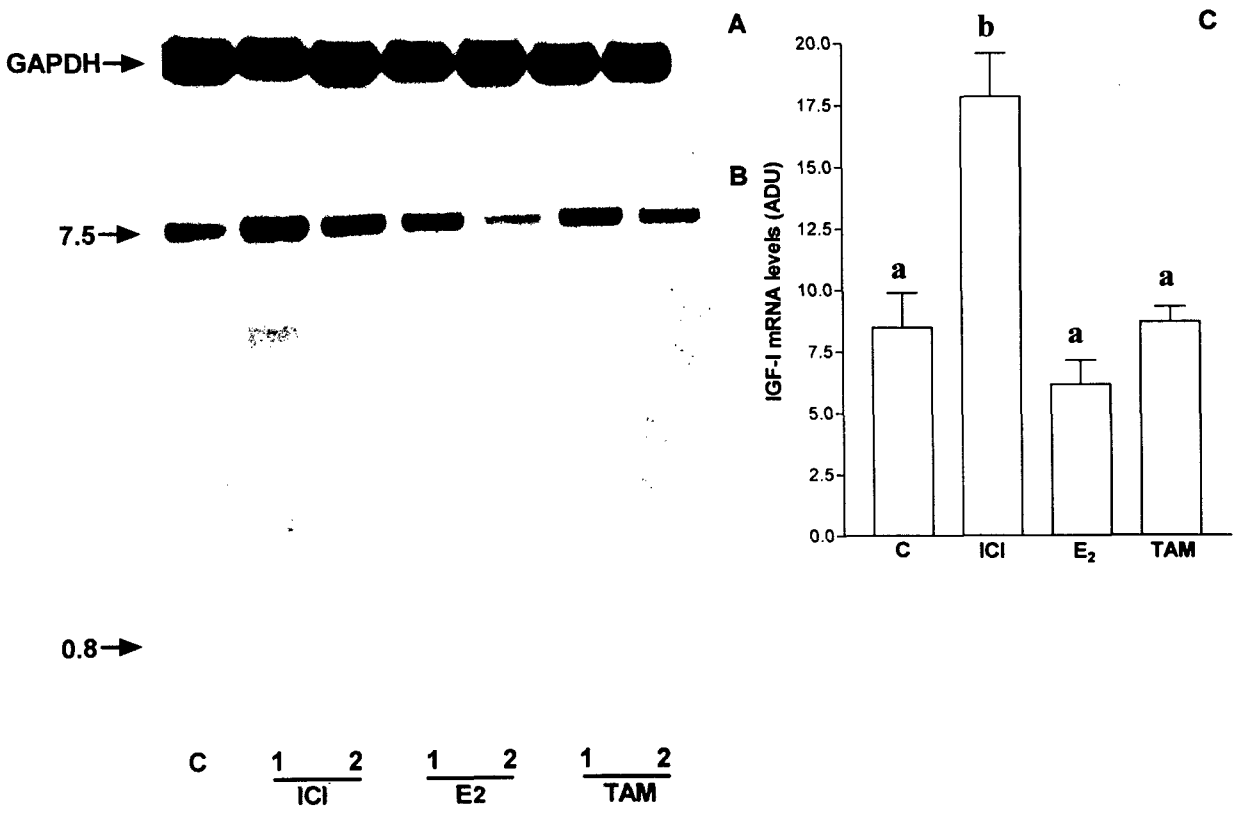

Ki-67 Index in Epithelium
(per 500 cells)
200
150
100
50
0
a
b
c
b
C
ICI
E2
TAM

FIGURE 2

FIGURE 3


GAPDH
7.5
0.8
C
1
2
ICI
1
2
E2
1
2
TAM
A
B
C
IGF-I mRNA levels (ADU)
20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
a
b
a
a
C
ICI
E2
TAM

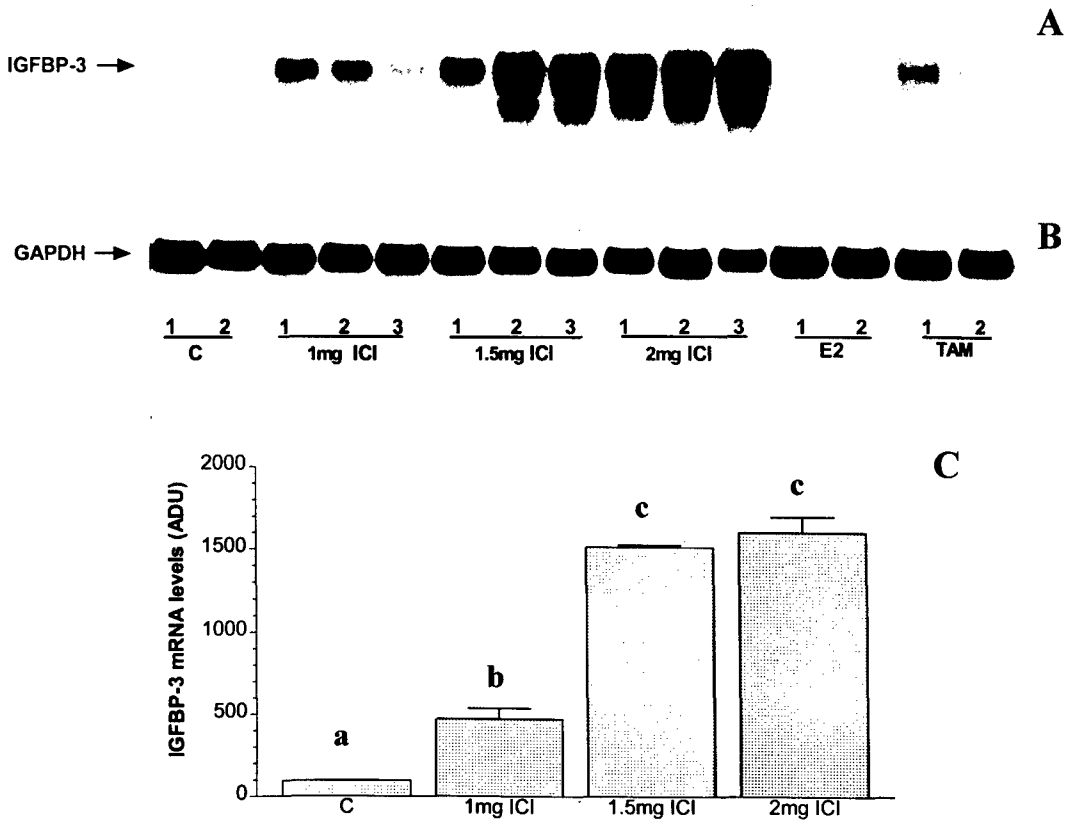

A
IGFBP-3
B
GAPDH
1 2
C
1 2 3
1mg ICI
1 2 3
1.5mg ICI
1 2 3
2mg ICI
1 2
E2
1 2
TAM
C
IGFBP-3 mRNA levels (ADU)
2000
1500
1000
500
0
a
b
c
c
C
1mg ICI
1.5mg ICI
2mg ICI

FIGURE 4

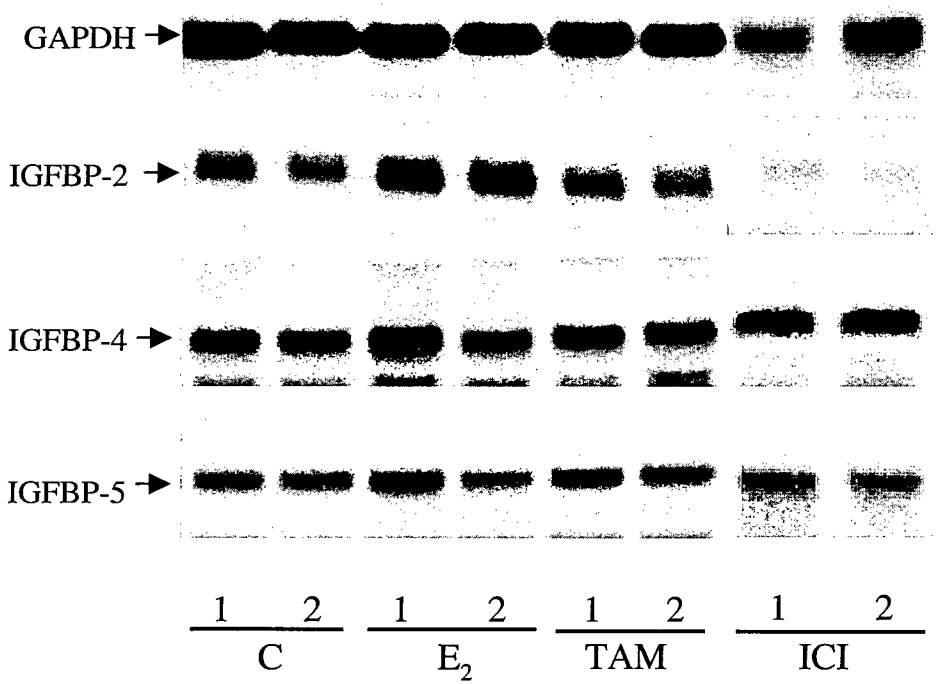


FIGURE 5

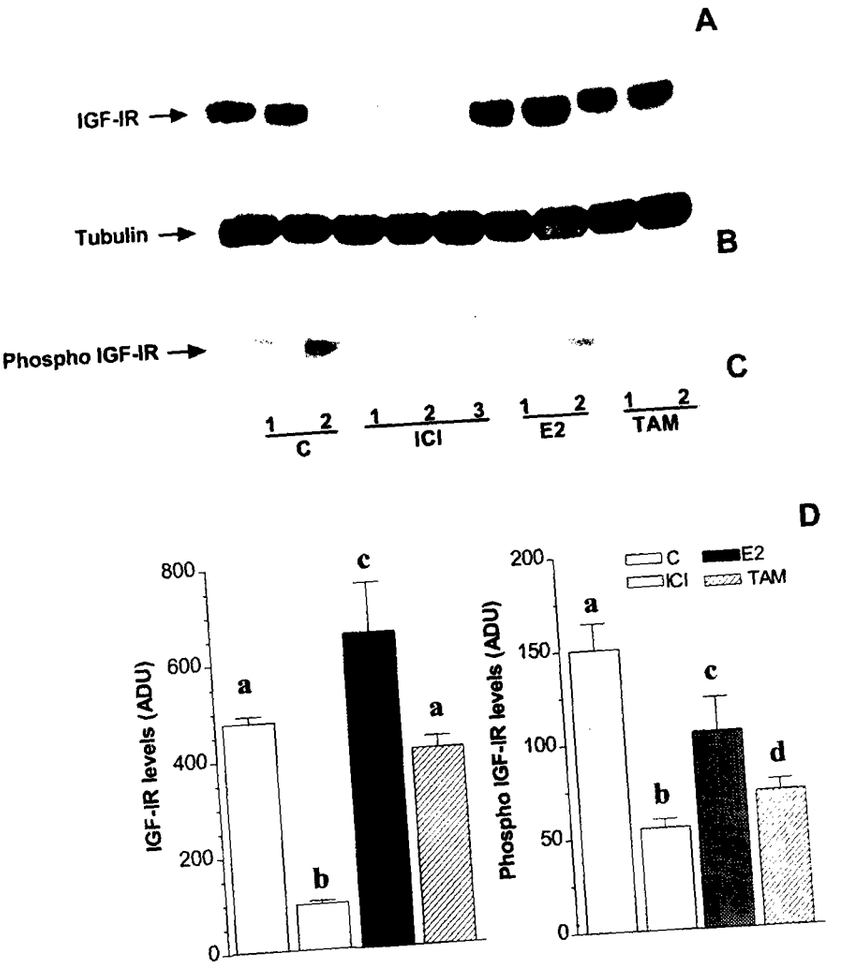

A
IGF-IR
Tubulin
B
Phospho IGF-IR
C
1 2
C
1 2 3
ICI
1 2
E2
1 2
TAM
D
IGF-IR levels (ADU)
800
600
400
200
0
a
b
c
a
Phospho IGF-IR levels (ADU)
200
150
100
50
0
C
E2
ICI
TAM
a
b
c
d

FIGURE 6

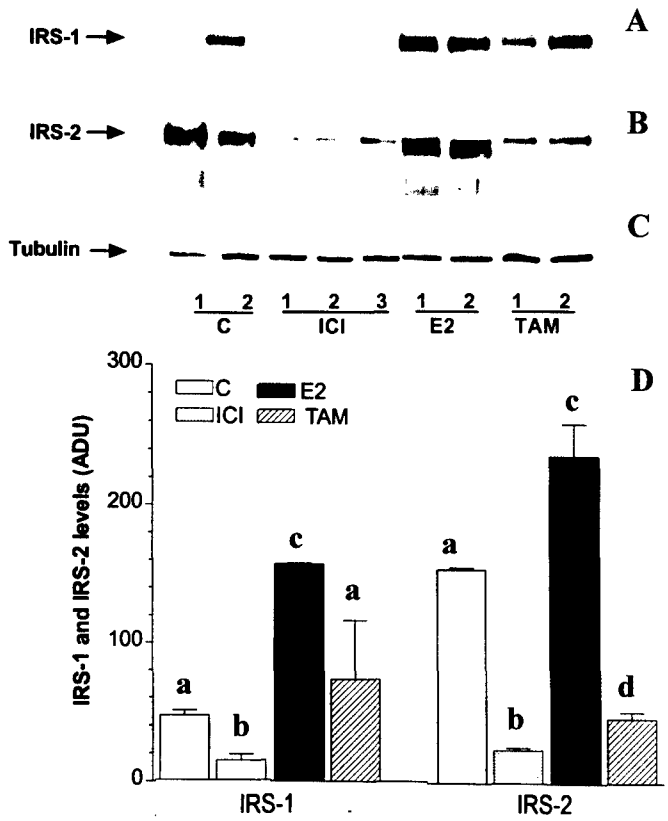

IRS-1
IRS-2
Tubulin
A
B
C
1 2
C
1 2 3
ICI
1 2
E2
1 2
TAM
D
C
E2
ICI
TAM
IRS-1 and IRS-2 levels (ADU)
300
200
100
0
a
b
c
a
a
b
c
d
IRS-1
IRS-2

FIGURE 7

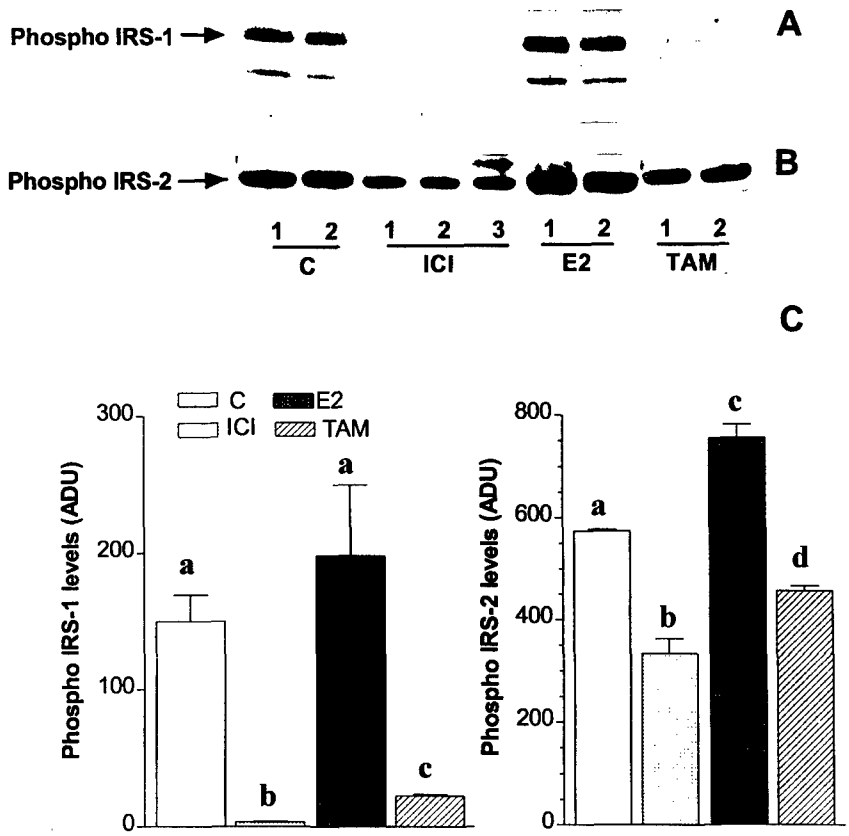

Phospho IRS-1
A
Phospho IRS-2
B
1 2
C
1 2 3
ICI
1 2
E2
1 2
TAM
C
C
E2
ICI
TAM
Phospho IRS-1 levels (ADU)
300
200
100
0
a
b
a
c
Phospho IRS-2 levels (ADU)
800
600
400
200
0
a
b
c
d

FIGURE 8

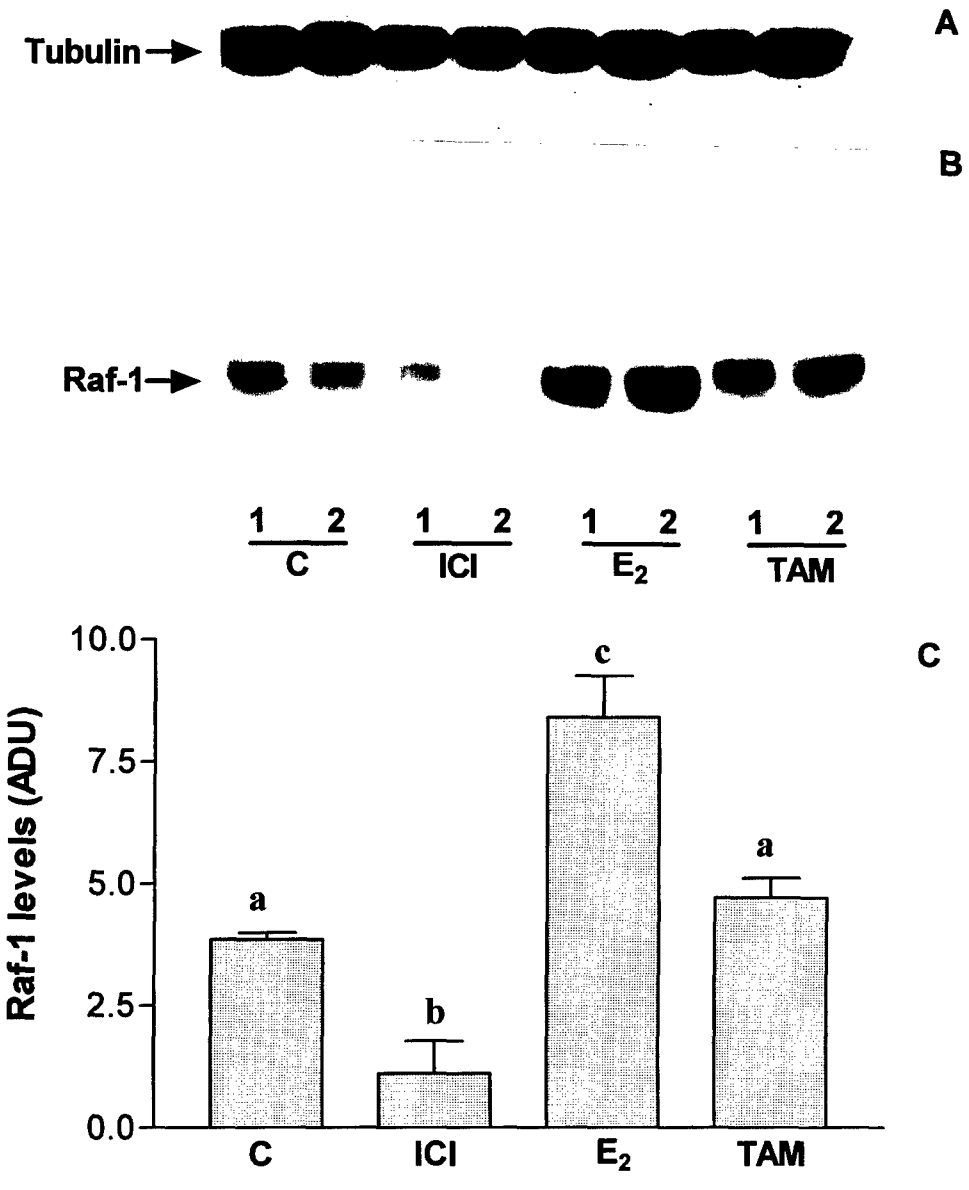

Tubulin
A
B
Raf-1
1 2
C
1 2
ICI
1 2
E2
1 2
TAM
C
Raf-1 levels (ADU)
10.0
7.5
5.0
2.5
0.0
a
b
c
a
C
ICI
E2
TAM

FIGURE 9

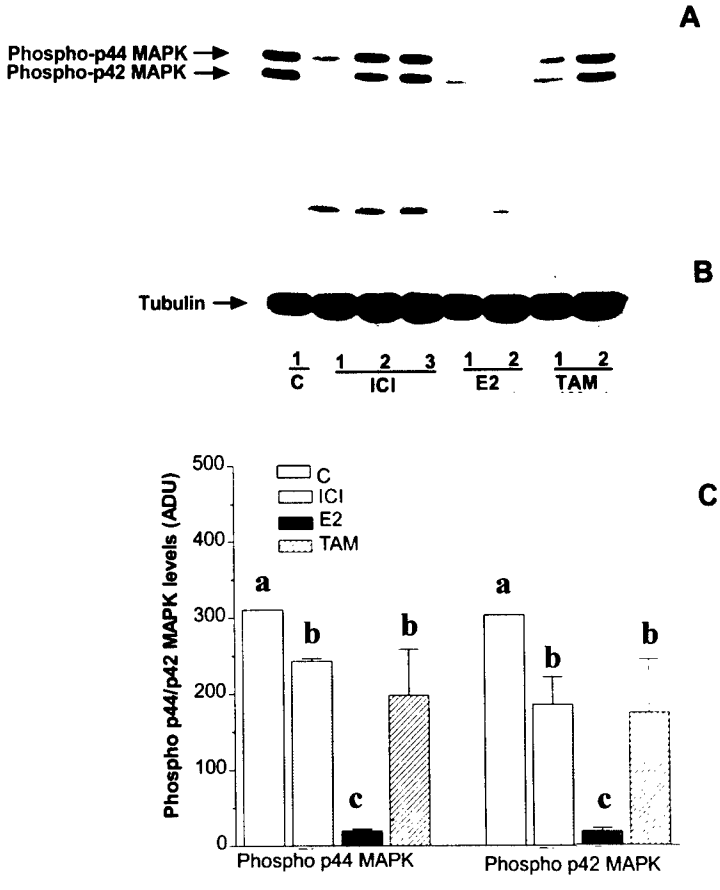

A
Phospho-p44 MAPK
Phospho-p42 MAPK
B
Tubulin
1
C
1 2 3
ICI
1 2
E2
1 2
TAM
C
500
400
300
200
100
0
Phospho p44/p42 MAPK levels (ADU)
C
ICI
E2
TAM
a
b
b
c
a
b
b
c
Phospho p44 MAPK
Phospho p42 MAPK

FIGURE 10

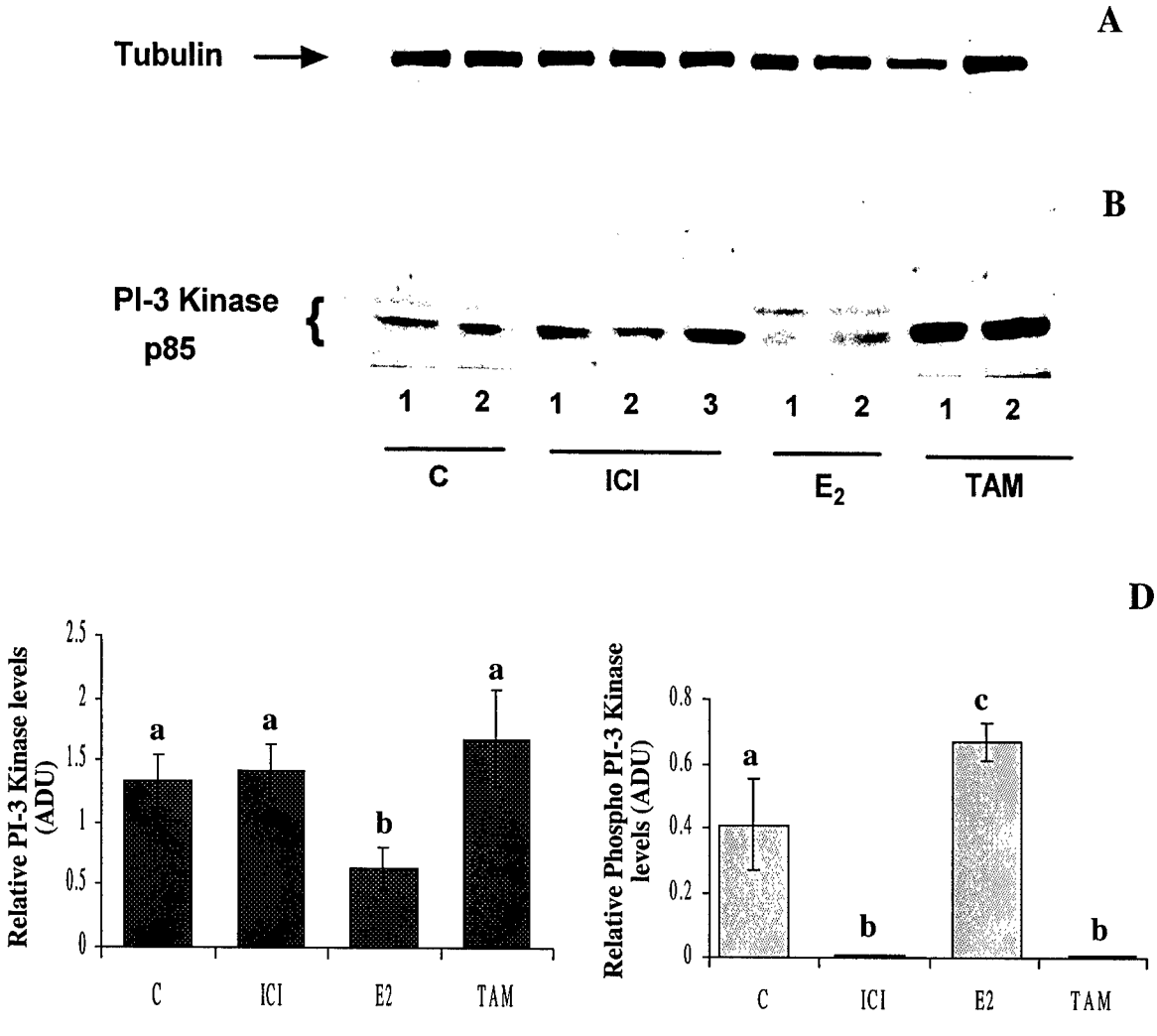

A
Tubulin
B
PI-3 Kinase
p85
1 2 1 2 3 1 2 1 2
C
ICI
E2
TAM
D
Relative PI-3 Kinase levels (ADU)
2.5
2
1.5
1
0.5
0
a a b a
C ICI E2 TAM
Relative Phospho PI-3 Kinase levels (ADU)
0.8
0.6
0.4
0.2
0
a b c b
C ICI E2 TAM

FIGURE 11

Characterization of a novel tamoxifen-induced cDNA (UO-44) in the rat uterus and ovary

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Running Title: Cloning of tamoxifen-induced cDNA

Abstract

A novel cDNA, UO-44, has been isolated from tamoxifen-induced rat uterine cDNA libraries using differential display techniques. UO-44 cDNA is 2282 bp in length and encodes a 607-amino acid protein of calculated molecular weight 68639 Da. UO-44 protein contains two CUB domains, a zona pellucida domain, hydrophobic and anchor transmembrane regions. UO-44 transcripts are abundant in the uterus and ovary where UO-44 gene expression is strongly induced by estrogens, tamoxifen and growth hormone while the pure antiestrogen ICI 182780 is inhibitory. *In situ* hybridization revealed that UO-44 gene expression is confined to the luminal and glandular epithelial cells of the uterus and to granulosa cells of average size ovarian follicles. Transfection studies showed UO-44 to be a membrane-associated protein. Since estrogens are potent stimulators of follicle development and uterine luminal epithelial cell growth *in vivo*, UO-44 protein may serve as a mediator of the effect of estrogenic hormones in inducing epithelial proliferation and differentiation in these tissues.

Introduction

It is clear that the female genital tract, including the uterus, exhibits considerable control over the ability of a conceptus to develop (1,2). The uterus expresses and secretes a number of growth factors (3-8) and other regulatory polypeptides (7,9) in response to ovarian steroid hormones. These polypeptides are thought to play a part in directing or limiting the growth and development of the uterus. Estrogens promote the growth, differentiation and remodelling of the uterus during the estrus cycle and pregnancy (10-12). They modulate the expression of genes involved in the regulation of cell growth and differentiation including EGF, IGF-I and their receptors (5,13-20). Preovulatory ovarian estrogen secretion is important for uterine cellular proliferation and epithelial differentiation during early stages of pregnancy (21).

The CUB domain is an extracellular domain of approximately 110 residues which is found in functionally diverse (mostly developmental) proteins. Proteins that contain the CUB domain include mammalian complement subcomponents C1s/C1R (22,23); hamster serine protease, Casp (24,25); and mammalian complement-activating component of Ra-reactive factor (RARF) (26); vertebrate bone morphogenic protein 1 (BMP-1) (27-31); neuropilin (A5 antigen) (32-39); mammalian hyaluronate-binding protein TSG-6, a serum- and growth factor-induced protein possibly involved in cell-cell and cell-matrix interactions during inflammation and tumorigenesis (40); and mammalian spermadhesins (24,41,42). Bovine acidic seminal fluid contains a CUB domain and belongs to the spermadhesin family. It functions both as a mitogen and growth factor *in vitro* (43) and a stimulator of progesterone secretion in cultured ovarian cells (43).

The antiestrogen drug tamoxifen improves the survival of women with breast cancer and has proved to be clinically useful for the treatment of metastatic estrogen receptor positive tumours (44). However, long-term administration of tamoxifen has been reported to be associated with an increased risk of endometrial cancer in post-menopausal women (45). Tamoxifen causes endometrial thickening in some postmenopausal women. Over 40% of women on tamoxifen had an endometrium > 8 mm thick compared with only 5% of control women on placebo (46). It has been suggested that the estrogenic effect of tamoxifen on the atrophic postmenopausal endometrium causes hyperplasia that may progress to atypia and cancer in a manner similar to that seen with estrogen replacement therapy. To identify additional tamoxifen- and estradiol-regulated genes in the uterus, differential display was used to examine the transcript expression profile of the ovariectomized uterus under conditions of tamoxifen supplementation. A novel tamoxifen-induced cDNA, UO-44, was isolated. By virtue of its activation by growth hormone, estradiol and tamoxifen, tissue-specific expression and localization on the cell membrane, UO-44 protein may be important in normal and neoplastic uterine and ovarian growth. It may potentially serve as a biomarker for uterine and ovarian cancer, and aid in their diagnosis and treatment.

Materials and Methods

Animals and drug administration. Animal experiments were approved by the local Animal Care Committee. We assessed the *in vivo* effects of the pure antiestrogen ICI 182780, tamoxifen and estrogens on UO-44 gene expression in the ovaries and uteri of a rat model. Intact, hypophysectomized (Hypox) or ovariectomized (OVX) female Sprague-Dawley rats, 50 days old at the beginning of the experiments, were obtained from Charles River, Quebec. OVX and Hypox animals were used in these experiments 2 weeks after ovariectomy and hypophysectomy, respectively. To study changes in UO-44 gene expression, uteri and ovaries were removed from rats at various stages of the estrous cycle as determined by vaginal smears. To study the effect of growth hormone on UO-44 gene expression, Hypox rats were daily injected with IP 1 μg recombinant human growth hormone (GH) (Genentech) per gram body weight for 21 days. To study the effect of estradiol and progesterone on UO-44 gene expression, groups of ovariectomized rats were implanted with 0.5 cm, 1.0 cm or 1.5 cm silastic tubes (0.04 in. ID, Dow Corning, Michigan) containing 17-ß estradiol or progesterone (Sigma) on the back of their necks. Control rats experienced the same surgical implantation with empty silastic tubes. Based on previously published work (47), the released rate of 17 ß-estradiol from silastic implants was documented to be 2.4 μg/cm/day. Tamoxifen (Sigma) was dissolved in castor oil at a concentration of 1 mg/ml. Female rats daily received either 200 μg or 400 μg Tamoxifen per kg BW via sc injections. Preformulated ICI 182780 (Zeneca Pharmaceuticals) was supplied at a concentration of 50 mg/ml in castor oil solution. Female Sprague-Dawley rats received weekly sc injections of castor oil alone, 1, 1.5 or 2 mg of ICI 182780 per kg BW for 3 weeks. At the end of the experiments, animals were sacrificed by carbon dioxide exposure. The uteri and ovaries were excised, trimmed, weighed and snap-frozen in liquid nitrogen and stored at -70 ^{0}C for RNA extraction. Part of the uterus and one

ovary were embedded in OCT for *in situ* hybridization studies.

To determine UO-44 gene expression during the estrous cycle, uteri were collected from rats at different stages of the cycle which were ascertained by examining vaginal smears.

mRNA differential display: Differential display was performed using RNA from tamoxifen-treated ovariectomized rat uteri and from untreated ovariectomized uteri according to the protocol supplied with the RNAmap^{TM} kit (GeneHunter Corp., Nashville, TN). Briefly, 5 μg of DNAse I-treated total RNA were reverse transcribed with $T_{12}M^n$ (where n may be G, A, T, or C), followed by PCR amplification in the presence of [α-^{33}P]dATP (NEN) using the corresponding $T_{12}M_n$ primer, downstream, and one of the arbitrary primers supplied with the kit, AP_1-AP_5, upstream. The PCR-amplified fragments were separated on 6% denaturing polyacrylamide gel. The gel was dried and exposed to Kodak XAR film with intensifying screens at -70 ^{0}C, and cDNA representing differentially expressed mRNAs were excised from the dried gels for reamplification. Reamplified cDNA fragments were used as probes in Northern blots to verify their differential expression in uteri. Desired fragments were used to screen a rat uterus cDNA library.

cDNA library construction: Ten μg of poly A^+ RNA derived from ovariectomized-tamoxifen-treated rat uterus was used to construct a unidirectional cDNA library in the vector pcDNA3.0 (Invitrogen, Carlsbad, CA) designed for expression in mammalian cells using the CMV promoter. cDNA was primed using the unidirectional Not "T" primer so giving inserts in the correct orientation for expression. Double stranded cDNA was size enriched and transformed into TOP10F' cells after ligation into the vector. The 320 bp probe of rat UO-44 cDNA was used to screen this rat uterine cDNA library as described (48). Clones identified by this probe were isolated

and sequenced by the Sanger dideoxy chain termination method and their nucleotide sequences were compared with those deposited in the GenBank and EMBL databases.

***In situ* hybridization**: For mRNA *in situ* hybridization, recombinant plasmid pcDNA3.0 containing a 500-bp UO-44 fragment (nucleotide 1780 to 2280 of the UO-44 sequence, GenBank accession number AF022147) was linearized to generate sense and antisense digoxigenin-labeled RNA probes using Dig RNA Labeling kit (Boehringer Mannheim). Serial 7-8 μm OCT-frozen sections were heated for 2 min at 50 ^{0}C and dried for 30 min. Prehybridization, hybridization, posthybridization and immunological detection were performed according to the manufacturer's protocol. The sections were counterstained with haematoxylin.

Northern Blot: Total RNA was isolated from indicated tissues of female rats as described (49). Northern blots were performed on total RNA and blots were hybridized with rat UO-44 or human GAPDH (ATCC) cDNAs as previously described (49). mRNA levels were determined by densitometric scanning of autoradiographs.

Stably transfected MCF-7 cell lines: The entire coding region of UO-44 cDNA was cloned into the mammalian expression vector pcDNA3.1/His (Invitrogen, Carlsbad, CA) to create the UO-44-pcDNA3.1/His expression vector. The UO-44-pcDNA3.1/His sequence was confirmed by sequencing. MCF-7 cells were seeded at a density of 2 x 10^5 in 100 mm culture dishes in 90% α-MEM (Life Technologies, Inc.) containing 10% FCS with Garamycin 24 h prior to transfection. Cells were transfected with 5 μg of UO-44-pDNA3.1/His DNA or pDNA3.1/His control plasmid

DNA and 28 μl of Lipofectamine reagent (Life Technologies) following recommendations of the manufacturer. Forty-eight hours following transfection, cells were subcultured at 1:10 and replaced with growth medium containing 800 μg/ml G418 (Calbiochem, La Jolla, CA). After 4 weeks, clones were isolated, expanded and assayed for UO-44 expression by western blot analysis.

Western Analysis: To localize the UO-44 protein, controls and UO-transfected MCF7 cells were grown to 90% confluency. Plasma membrane-enriched subcellular fractions and cytosol were prepared by differential centrifugation as described previously (50). Plasma membrane and cytosolic proteins were subjected to western blot analysis as described (51). Blots were incubated with mouse anti 6-Histidine antibody (Epitope Tagging) Ab-1 (NeoMarkers, Union City, CA) (1:500 dilution) and horseradish peroxidase-conjugated donkey anti-mouse secondary antibody (1:7500). Blots were visualized with a chemiluminescent detection system (ECL, Amersham) and exposed to film for 10 sec to 45 sec.

Results

The cDNA (pDNA3.0) library prepared from tamoxifen-treated ovariectomized rat uterus was screened with the 320 bp probe isolated by differential display using conditions optimized for Northern blot analysis. Fourteen clones were isolated and purified. One clone containing a 2.2 kb insert was sequenced by the Sanger dideoxy chain termination method.

Comparison of the nucleotide sequence obtained against the non-redundant nucleotide database of GenBank established the cDNA as novel. The cDNA (GenBank accession no AF022147) contains 2282 bp and is full length. An initiator ATG codon (position 253) is followed by a single open reading frame of 607 amino acids with a calculated molecular weight of 68639 Da. The ATG initiation site is contained in the sequence for initiation by eukaryotic ribosomes described by Kozak (52). The open reading frame ends in a TGA terminator codon at position 2074 followed by 208 nucleotides in the 3' untranslated region.

The UO-44 amino acid sequence predicts a membrane protein with two transmembrane helices. The hydrophobic transmembrane is 13 amino acids in length and is located between amino acids 5 and 17, while the anchor transmembrane region is 19 amino acids in length and is located between amino acids 571 and 589. There is a putative transmembrane domain near the carboxyl terminus suggesting that UO-44 may function as an integral membrane-associated protein analogous to ebnerin or TGF betaglycan (53). UO-44 terminates in a short 19 amino acid polypeptide presumably positioned within the cytoplasm.

The protein was evaluated against the non-redundant protein database of GenBank (54). Within the first 265 amino acids of UO-44, two regions were identified that displayed similarity to the CUB motifs. Analysis of these regions using ProfileScan program confirms that the protein contains 2 CUB domains; the first begins at Cys-32 and the second at Cys-154. Block search (55) revealed that UO-44 also contains a zona pellucida domain at amino acids 435-459.

The distribution of UO-44 mRNA was examined by Northern analysis using tissues obtained from mature female rats. Transcription of the UO-44 gene was observed only in uterus and ovary. UO-44 mRNA levels in adipose tissue, mammary gland, liver, kidney, muscle, heart, stomach, small intestine, spleen, brain, pituitary, and muscle were undetectable, suggesting that the UO-44 gene may be expressed at a very low level or not at all in these tissues (Fig.1).

The UO-44 gene is expressed in the uterus of intact female rats. Its basal expression was greatly enhanced by both estradiol and tamoxifen. At the dose of 50 μg/rat/day, tamoxifen stimulated UO-44 gene expression but UO-44 mRNA levels returned to basal levels at the higher dose, 100 μg/rat/day (Fig. 2A). Estradiol was more potent than tamoxifen at inducing UO-44 mRNA accumulation. Maximal effects were obtained at the dose of 1.2 μg/rat/day (Fig. 2B). ICI 182780, on the other hand, was a very potent inhibitor of UO-44 gene expression, completely abolishing UO-44 gene expression in the uterus even in the presence of ovaries (Fig. 2C).

To determine if UO-44 gene expression fluctuates during the estrous cycle, total RNA derived from uteri of rats at different stages of the cycle were analyzed by Northern blotting. As shown in figure 3A, maximal expression of UO-44 occurred at estrus and proestrus stages coincident with estrogen-induced uterine cell proliferation. Low levels of UO-44 mRNA were detected during metestrus and

diestrus. A similar pattern of UO-44 expression was also observed in the ovaries at different stages of the estrus cycle although the changes were less pronounced (Fig. 3B).

Loss of UO-44 uterine expression was very rapid following ovariectomy. Six hours after removal of the ovaries, UO-44 mRNA levels dropped to 30% of controls and no UO-44 transcripts were detected 144 h post-ovariectomy (Fig. 4). Tamoxifen restored UO-44 gene expression in the ovariectomized uterus (Fig. 5) as did estradiol and diethylstilbestrol which strongly stimulated UO-44 gene expression, such that UO-44 mRNA levels were 3- to 4-fold higher even than in uteri from ovary-intact rats. ICI 182780 potently attenuated estradiol-induced UO-44 gene expression (Fig. 5). Progesterone had no effect on UO-44 gene expression. There was a positive correlation between: (1) levels of UO-44 mRNA and uterine weights and (2) the potency of compounds to increase uterine weight and their ability to induce UO-44 gene expression.

To examine if UO-44 gene expression in the uterus and ovary is also under growth hormone regulation, Northern blot analysis was performed using uteri and ovaries derived from hypophysectomized rats treated with vehicle or recombinant human growth hormone. As shown in figure 6, hypophysectomy caused a reduction in uterine weight which was coincident with the disappearance of UO-44 mRNA. UO-44 gene expression in the uterus was effectively restored by estradiol, tamoxifen, growth hormone and diethylstilbestrol in hypophysectomized rats. Hypophysectomy, on the other hand, had very little or no effect on UO-44 gene expression in the ovary. Blockade of estrogenic stimulation by a pure antiestrogen ICI 182780 led to a dramatic reduction in UO-44 mRNA in the ovaries (Fig. 6B).

In situ hybridization was performed on sections of rat uterus using an antisense RNA probe specific for UO-44 to localize its expression. UO-44 mRNA was only detected in the luminal epithelial cells and glandular population (Fig. 7A and 7B). No staining was seen in smooth muscle cells. Hybridization with sense UO-44 RNA probe showed no background staining (Fig. 7C). *In situ* hybridization with UO-44 sense and antisense probes was also performed on sections of ICI 182780-treated uterus. Hybridization with anti-sense UO-44 gave very weak signal staining (Fig. 7D).

In situ hybridization with antisense RNA probe for UO-44 expression in rat ovaries revealed that UO-44 mRNA was detected only in granulosa cells and that not all follicles expressed UO-44 (Fig. 8A and 8B). High levels of UO-44 expression were observed in the granulosa cells of medium size follicles (Fig. 8A). Low to moderate UO-44 expression was detected in granulosa cells of small and large follicles (Fig. 8A). Furthermore, lack of uniform UO-44 gene expression among granulosa cells within the same follicle was noted (Fig. 8A). A control sense UO-44 probe produced no background staining in ovarian tissue (Fig. 8C). Very weak staining signals were detected in sections of ovaries from rats treated with ICI 182780 (Fig. 8D).

To further demonstrate the subcellular localization of UO-44 protein, human breast cancer MCF-7 cells were transfected with a mammalian expression vector containing full length UO-44 cDNA (UO-44 pcDNA3.1/His) or control pcDNA3.1/His vector. As shown in Figure 9, 6-Histidine antibody recognized a protein of approximately 68-69 kDa in plasma membrane-enriched subcellular fractions of UO-44 transfectants but not in the cytosol. No protein of identical size was detected in mock-transfected cells.

Discussion

In the course of studies to elucidate the molecular basis for the association of estradiol and tamoxifen treatment with increased risk of endometrial carcinoma, we have isolated an estradiol- and tamoxifen-regulated cDNA, UO-44, using differential display and cDNA library screening. The UO-44 cDNA contains 2282 bp and encodes for 607 amino acids with a calculated molecular weight of 68639 Da. Two CUB domains are present within the first 265 amino acids of the UO-44 protein which also contains a zona pellucida domain at the amino acid 435-459, a hydrophobic transmembrane region and an anchor transmembrane region. Transfection studies revealed that UO-44 is a membrane-associated protein, in agreement with the above prediction.

UO-44 transcripts are detected in the uterus and ovary. UO-44 mRNA is undetectable in adipose tissue, mammary gland, liver, kidney, muscle, heart, stomach, small intestine, spleen, brain, pituitary, and muscle suggesting that the OU-44 gene may be expressed at a very low level or is silent in these tissues. In the uterus of ovary-intact rats, UO-44 gene is expressed in the uterus and can be stimulated by estradiol, growth hormone or tamoxifen while a pure antiestrogen ICI 182780 completely abolishes UO-44 gene expression. UO-44 gene expression in rat uteri decreases rapidly and becomes undetectable following ovariectomy. Estradiol, diethylstilbestrol and tamoxifen but not progesterone efficiently restore UO-44 gene expression in the uteri of ovariectomized rats, while ICI 182780 blocks estradiol-induced UO-44 gene expression. Growth hormone is as effective as estrogen agonists in restoring UO-44 expression in uteri from hypophysectomized rats. Taken together, these data indicate that UO-44 gene expression is tissue-specific and regulated not only by estrogen agonists but also by growth hormone. In all cases, UO-44 expression is positively correlated with uterine growth.

In situ hybridization studies reveal that UO-44 gene expression is confined to the luminal and glandular epithelial cells of the uterus and granulosa cells of medium size follicles. High levels of UO-44 gene expression are found only in granulosa cells of medium size follicles but not in small and large follicles within the same ovary suggesting that UO-44 protein may be involved in follicular growth and maturation.

On the basis of homology to other proteins, there is 87% (at the nucleic acid level) and 88% (at the amino acid level) homology between rat UO-44 cDNA and UTCZP (uterine cub motif zona pellucida motif) cDNA (56). In contrast to the mouse UTCZP gene (a cDNA cloned from pregnant mouse uterus which is expressed only in the uterus during late pregnancy), rat UO-44 is expressed in non-pregnant and pregnant uteri. Chen et al. (57) recently reported a rat ERG1 cDNA which shares 99% homology with rat UO-44 cDNA. Like the UO-44 gene, the ERG1 gene is strongly induced in rat uterus by estradiol treatment and its expression is restricted to surface epithelium. In addition, UO-44 mRNA is also detected in granulosa cells of ovaries.

The biological function of UO-44 in the uterus and ovaries is unclear. The UO-44 gene product contains several motifs including a zona pellucida domain in the carboxyl terminal region. This domain appears to be involved in sperm-binding function and sperm-egg recognition (53). In the follicle, UO-44 expression is detected in the granulosa cells surrounding the egg. This pattern of expression is similar to the glycoproteins of the extracellular matrix surrounding the oocytes (53). Unlike the UTCZP gene (56) whose expression is temporal and restricted to the gravid uterus, UO-44 transcripts were not only detected in the uterus during pregnancy but also in non-pregnant uteri. UO-44 mRNA is readily detectable in the uterus of mature female rats. Loss of UO-44 gene expression following ovariectomy was completely restored by estradiol and tamoxifen. The

expression of UO-44 in the uteri and ovaries suggests that UO-44 protein may play other roles in these tissues besides events that transpire during pregnancy as proposed by Kasik (56). Since estrogens are potent growth stimulators for uterine luminal epithelial cells *in vivo*, the increase in UO-44 expression in uteri and ovaries may be associated with cellular proliferation in these tissues.

UO-44 contains 2 CUB domains. The biological function of CUB domains in UO-44 is unknown. It is possible that the CUB domains in UO-44 are involved in cell adhesion as reported for a calcium-independent cell adhesion molecule that functions during the formation of certain neural circuits (33,34). If this is the case, then UO-44 expression in uterine epithelial cells in response to increasing levels of circulating estrogens during early pregnancy may facilitate the attachment of embryos to the uterine wall. UO-44 expression in the granulosa cells surrounding the egg may serve as an adhesion molecule for cell-cell interaction and as a target for egg-sperm recognition as described for mammalian spermadhesins (42).

Maximal expression of UO-44 occurred at the estrus and proestrous stages of the ovarian cycle coincident with estrogen-induced uterine cell proliferation in contrast to low levels during metestrus and diestrus. In rodents, circulating levels of estrogen are high in proestrous and low in diestrous. Concomitant with this rise and fall in circulating estrogen levels, there is a rise and decline in UO-44 mRNA levels in the uterus. This pattern of expression during the reproductive cycle suggests that UO-44 might have a critical role in growth regulation. It is possible that UO-44 is an estrodiol-induced protein that is involved in cell-cell and cell-matrix interactions during estrogen-induced growth and tumorigenesis as described for mammalian hyaluronate-binding protein TSG-6 (40). Thus, it remains to be determined if UO-44 protein shares similar functions described for bovine acidic seminal fluid protein which contains a CUB domain and belongs to the spermadhesin family,

functioning both as a mitogen and growth factor *in vitro* and as a stimulator of progesterone secretion in cultured ovarian cells (43).

It is not known whether endometrial and ovarian cancer cells express UO-44 and whether the expression in such cells is regulated by estrogens and tamoxifen. If the UO-44 gene is over-expressed in these cancers and is regulated by estrogens, then the UO-44 protein can be used as a tumour-associated antigen with potential relevance to uterine and ovarian cancer immunotherapy. Being a tissue- and cell-specific membrane protein that is regulated by estrogen agonists, UO-44 is an ideal target protein for immunotherapy of uterine and ovarian cancers. Our data demonstrate that UO-44 gene expression in the uterus is a molecular marker that correlates well with the positive or negative uterotrophic effects of estrogen receptor antagonists and partial agonists. The characterization of UO-44 protein in uterine and ovarian cancer cells will open a new field of investigation that may lead to the development of therapeutic vaccines against uterine and ovarian cancers.

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Figure Legends

Figure 1. Northern blot analysis of UO-44 gene expression in female adult rat tissues. Total RNA derived from various tissues of an 80-day old female rat was subjected to northern blot. Blots were hybridized with UO-44 (**A**) and GAPDH (**B**) cDNAs. Tissues are: **Mg**: mammary gland; **Fa**: abdominal fat; **Mu**: red muscle; **Ov**: ovary; **He**: heart; **Lu**: lung; **Li**: liver; **Sto**: stomach; **Int**: small intestine; **Spl**: spleen; **Pi**: pituitary; **Br**: brain; **Ki**: kidney; and **Ut**: uterus.

Figure 2. Effects of tamoxifen, estradiol and ICI 182780 on uterine UO-44 gene expression and uterine weight. Ovary-intact rats were treated with indicated concentrations of tamoxifen (**TAM**), **A**, 17-ß estradiol (E_2), **B**, and ICI 182780 (**ICI**), **C**. Total RNA derived from uteri was subjected to northern blot. Blots were hybridized with GAPDH and rat UO-44 cDNAs. The effect of each treatment on uterine weight is shown. Data are expressed as the mean ± SEM.

Figure 3. Expression of UO-44 gene in rat uterus and ovaries at different stages of estrus cycle. Total RNA derived from uteri (**A**) or ovaries (**B**) of rats at different stages of the estrus cycle as indicated were analyzed by Northern blotting. Blots were hybridized with either GAPDH or UO-44 cDNAs.

Figure 4. Estradiol-dependent UO-44 gene expression. Ovariectomized rats were treated with 2.4 μg 17ß-estradiol per day. After 1 week of treatment, the estradiol implants were removed and the uteri were collected at the times indicated. Total RNA derived from uteri was subjected to northern blot. Blots were hybridized with GAPDH (**A**) and rat UO-44 (**B**) cDNAs. Densitometric scanning of the UO-44 bands is shown in (**C**). Data are expressed as the mean ± SEM.

Figure 5. Effects of tamoxifen, progesterone, ICI 182780, diethylstilbestrol, and estradiol on UO-44 gene expression in ovariectomized uterus. Ovariectomized rats were treated with vehicle (**C**), 400 μg tamoxifen per kg BW per day (**Ta**), 2.4 μg progesterone per day ($\mathbf{P_4}$), 1.5 mg ICI 182780 per kg BW per week (**ICI**), 2.4 μg 17-ß estradiol per day ($\mathbf{E_2}$), and 10 μg diethylstilbestrol per day (**DES** 10). Total RNA derived from uteri was subjected to northern blot. Blots were hybridized with rat UO-44 (**A**) and GAPDH (**B**) cDNAs. The effect of each treatment on uterine weight is shown in (**C**). Data are expressed as the mean ± SEM.

Figure 6. Effects of tamoxifen, ICI 182780, growth hormone, and estradiol on UO-44 gene expression in uterus and ovary of hypophysectomized rats. Hypophysectomized rats were treated with vehicle (**C**), 1 μg human growth hormone per gram BW (**GH**), 1.5 mg ICI 182780 per kg BW per week (**ICI**), 2.4 μg 17-ß estradiol per day ($\mathbf{E_2}$) and 400 μg tamoxifen per kg BW per day (**TAM**). Total RNA derived from uterus (**A**) and ovaries (**B**) was subjected to northern blot. Blots were hybridized with rat UO-44 or GAPDH cDNAs. The 18S and 28S bands served as loading controls.

Figure 7. *In situ* hybridization with antisense RNA probe for UO-44 expression in rat uterus. Low-power (**A**) and high-power (**B**) magnification showing UO-44 mRNA in luminal secretory epithelial cells and glandular epithelial cells. (**C**) sense control UO-44 probe showing no background staining in ovary intact rat uterus. (**D**) ICI 182780-treated uteri hybridized with anti-sense UO-44 showed very faint staining signal.

Figure 8. *In situ* hybridization with antisense RNA probe for UO-44 expression in rat ovaries. Low-power (**A**) and high-power (**B**) magnification showing UO-44 mRNA in follicles and

granulosa cells. UO-44 expression is not uniform. High levels of UO-44 expression were detected in the granulosa cells of medium size follicles. Moderate UO-44 expression was detected in granulosa cells of large and small follicles. (**C**) sense control UO-44 probe showing no background staining in ovarian tissue.(**D**) ICI 182780-treated ovary hybridized with anti-sense UO-44 showed very faint staining signal.

Figure 9. Subcellular localization of UO-44 protein. Human breast cancer MCF-7 cells were transfected with mammalian expression vector containing full length UO-44 cDNA (UO-44 pcDNA3.1/His) or control pcDNA3.1/His vector as described under "Materials and Methods". Plasma membrane-enriched subcellular fractions and cytosolic proteins were isolated and western blot analysis was performed as described under "Materials and Methods". Blots were incubated with mouse anti 6-Histidine antibody and horseradish peroxidase-conjugated donkey anti-mouse secondary antibody. Blots were visualized with a chemiluminescent detection system. Molecular weights of immunoreactive bands are shown. Clones 1 and 2 are mock transfectants; and UO-44-12 and UO-44-15 are UO-44 expressing clones.

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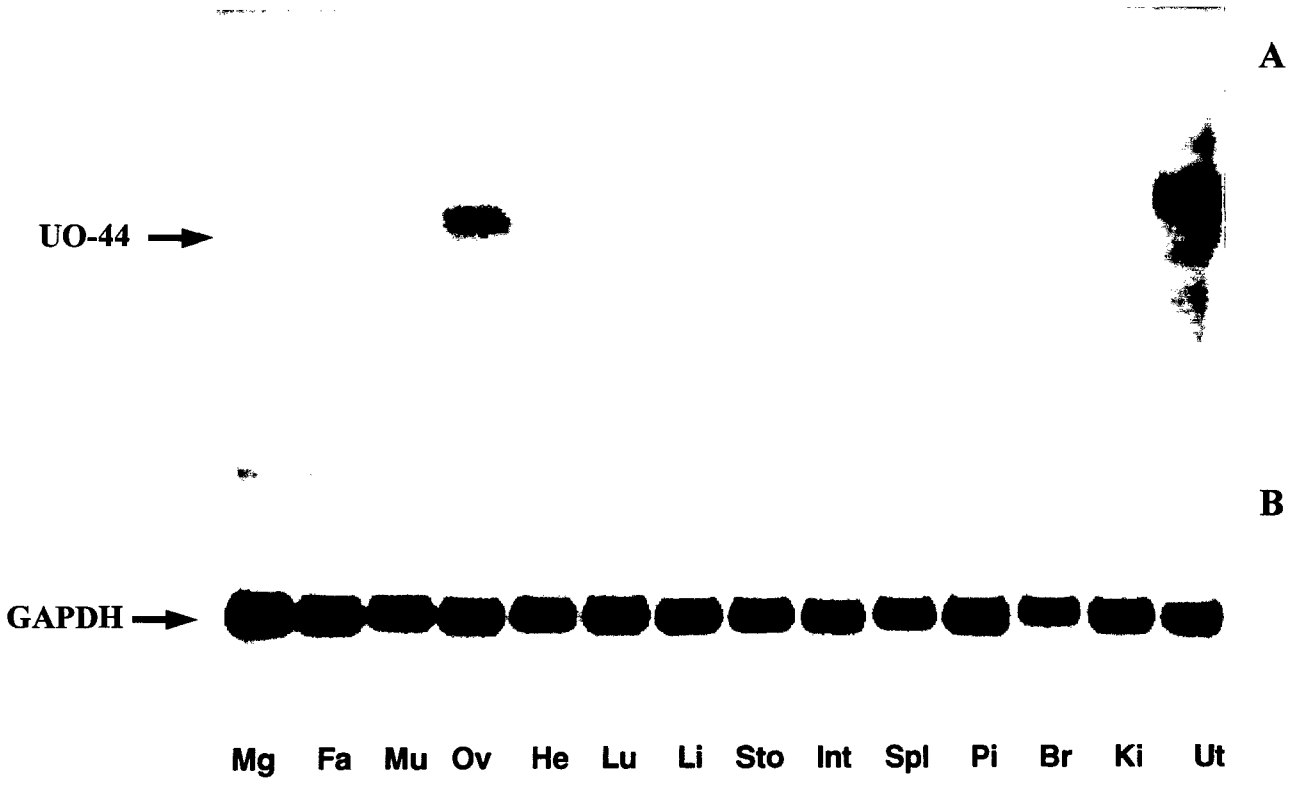
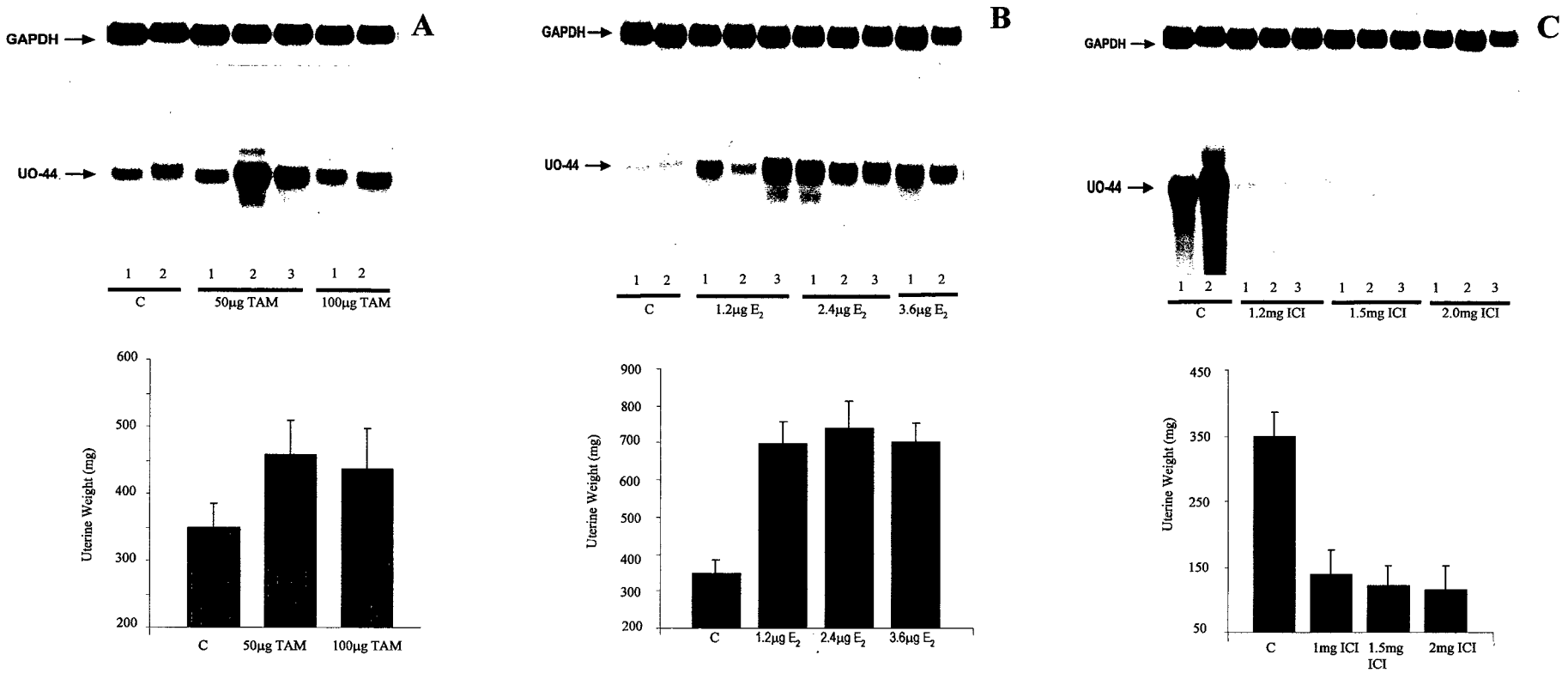


FIGURE 1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UO-44
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C
1.2μg E2
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3.6μg E2
Uterine Weight (mg)
900
800
700
600
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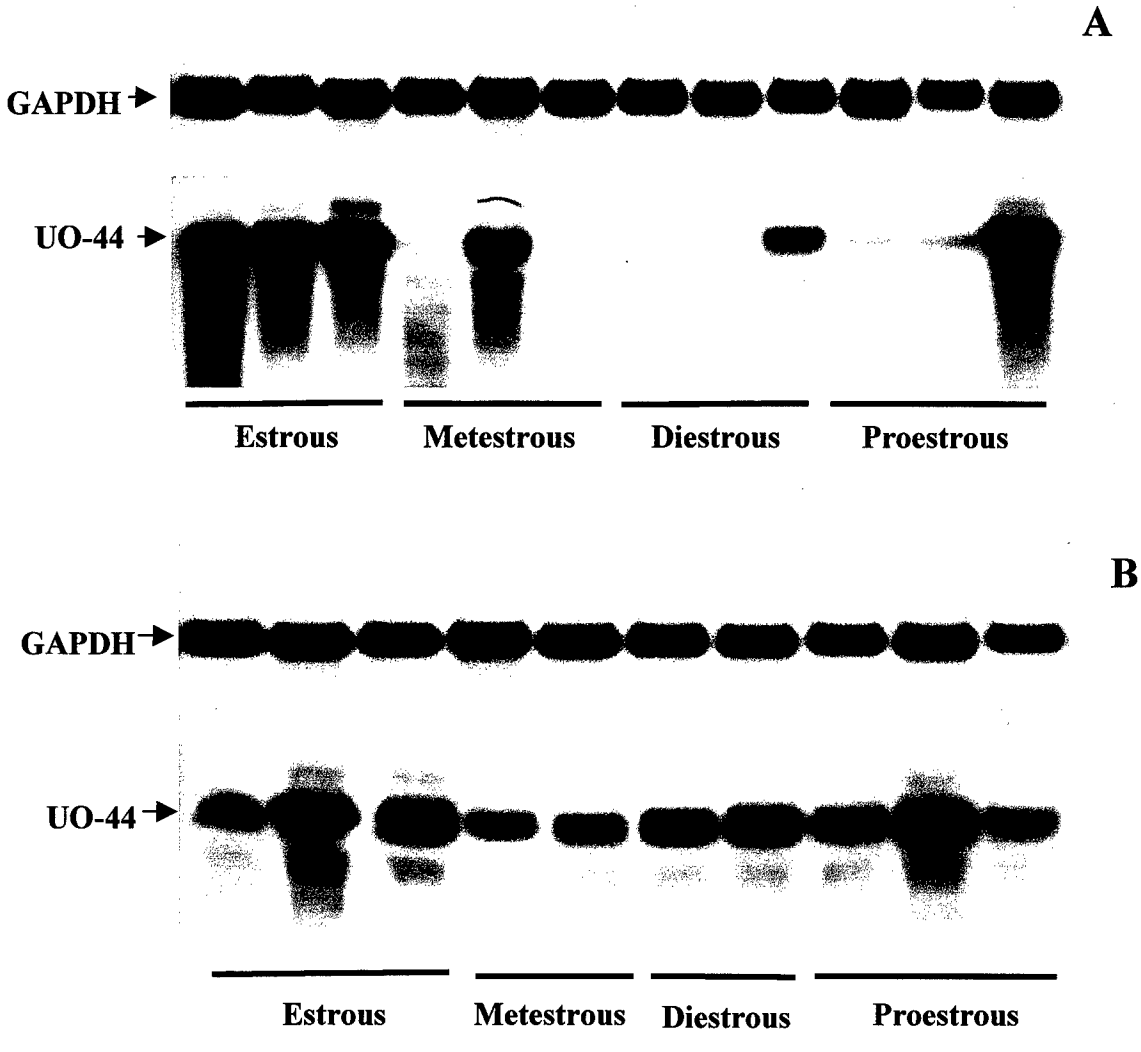
Figure 2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Proestrous

FIGURE 3

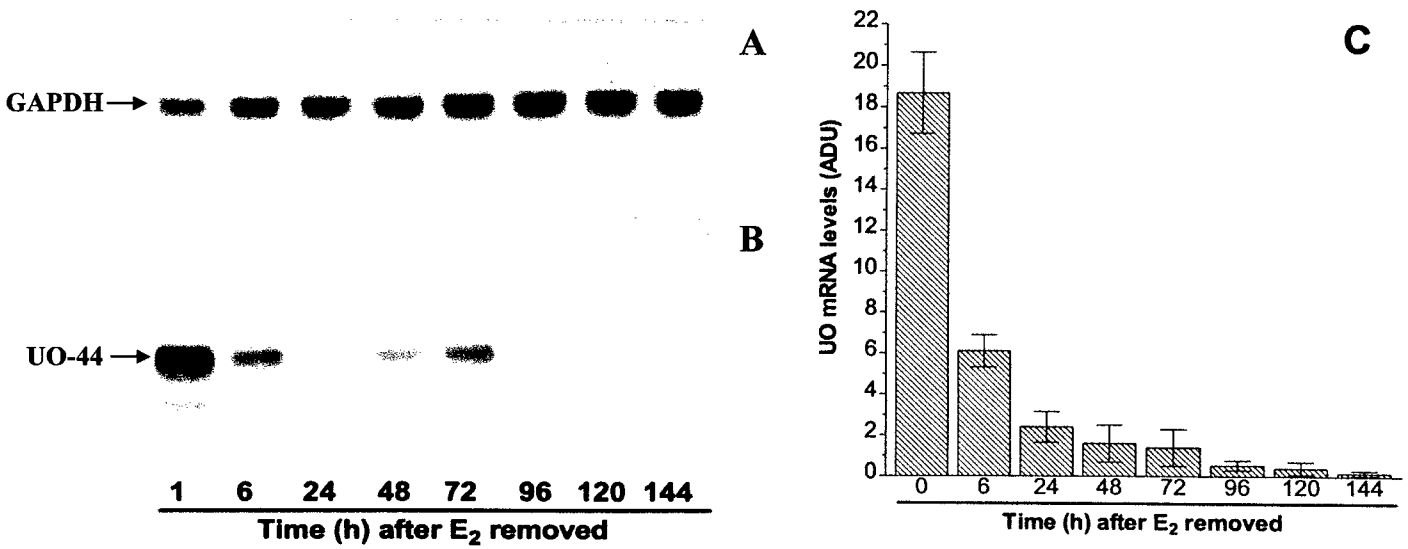
A
GAPDH
B
UO-44
1 6 24 48 72 96 120 144
Time (h) after E_2 removed
C
UO mRNA levels (ADU)
22
20
18
16
14
12
10
8
6
4
2
0
0 6 24 48 72 96 120 144
Time (h) after E_2 removed

Figure 4

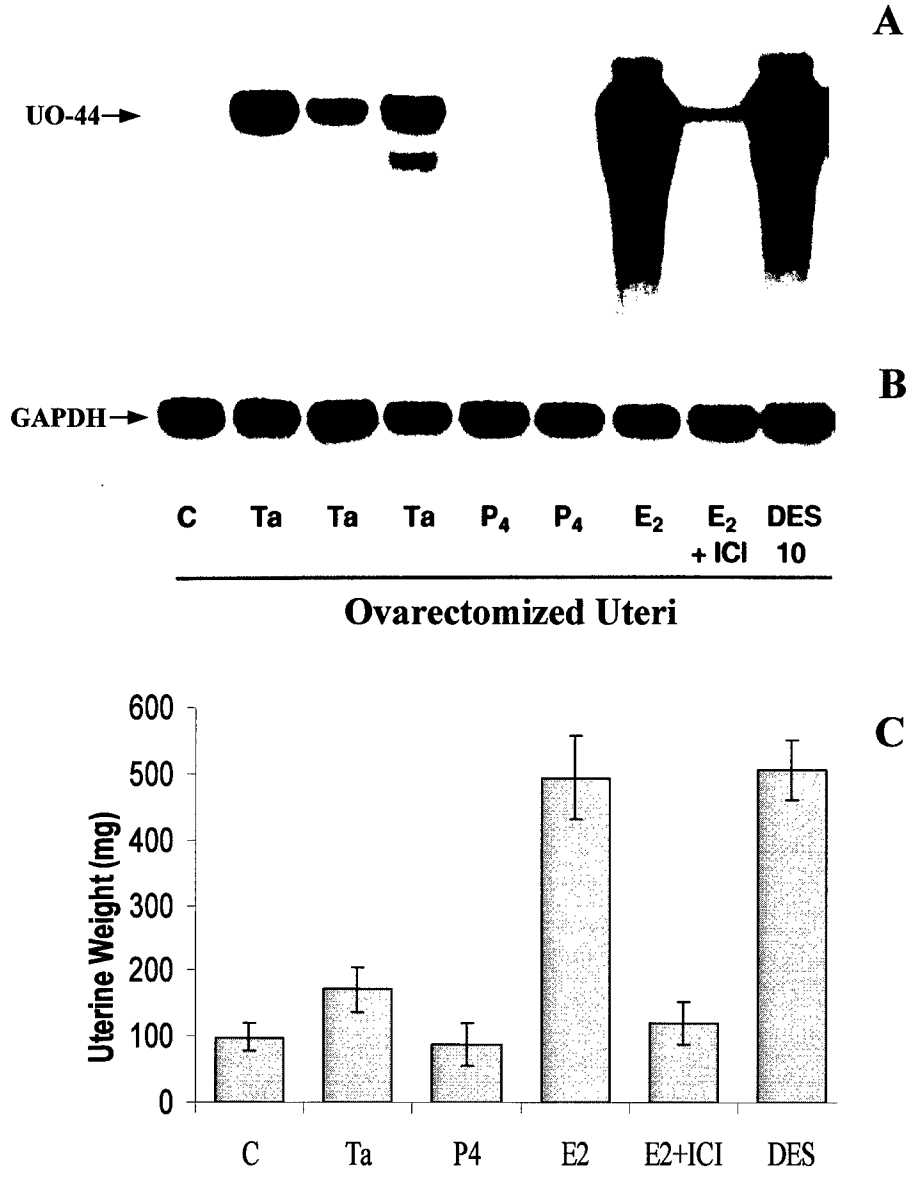
A
UO-44
B
GAPDH
C
Ta
Ta
Ta
P_4
P_4
E_2
E_2 + ICI
DES 10
Ovarectomized Uteri
C
600
500
400
300
200
100
0
Uterine Weight (mg)
C
Ta
P4
E2
E2+ICI
DES

FIGURE 5

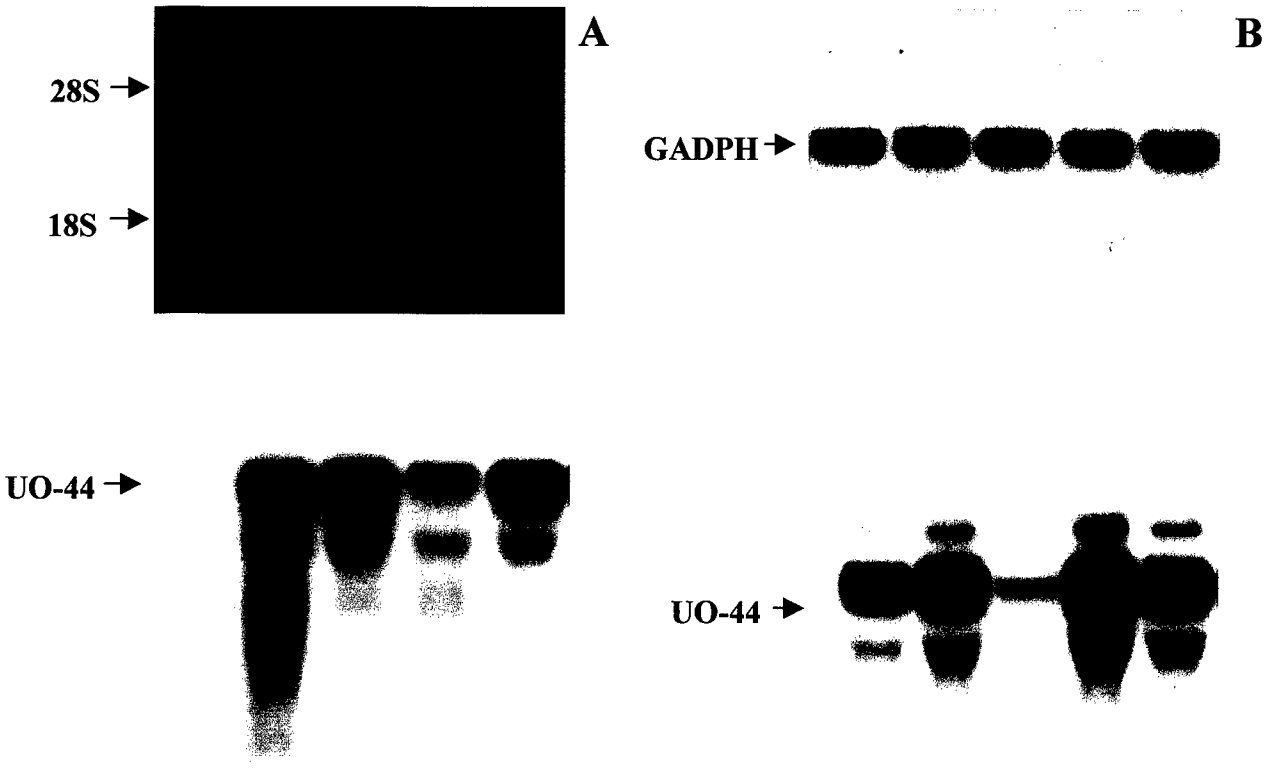
A
B
28S
18S
GADPH
UO-44
UO-44
C E2 TAM DES GH
Hypophysectomised Uteri
C GH ICI E2 TAM
Hypophysectomised Ovaries

FIGURE 6

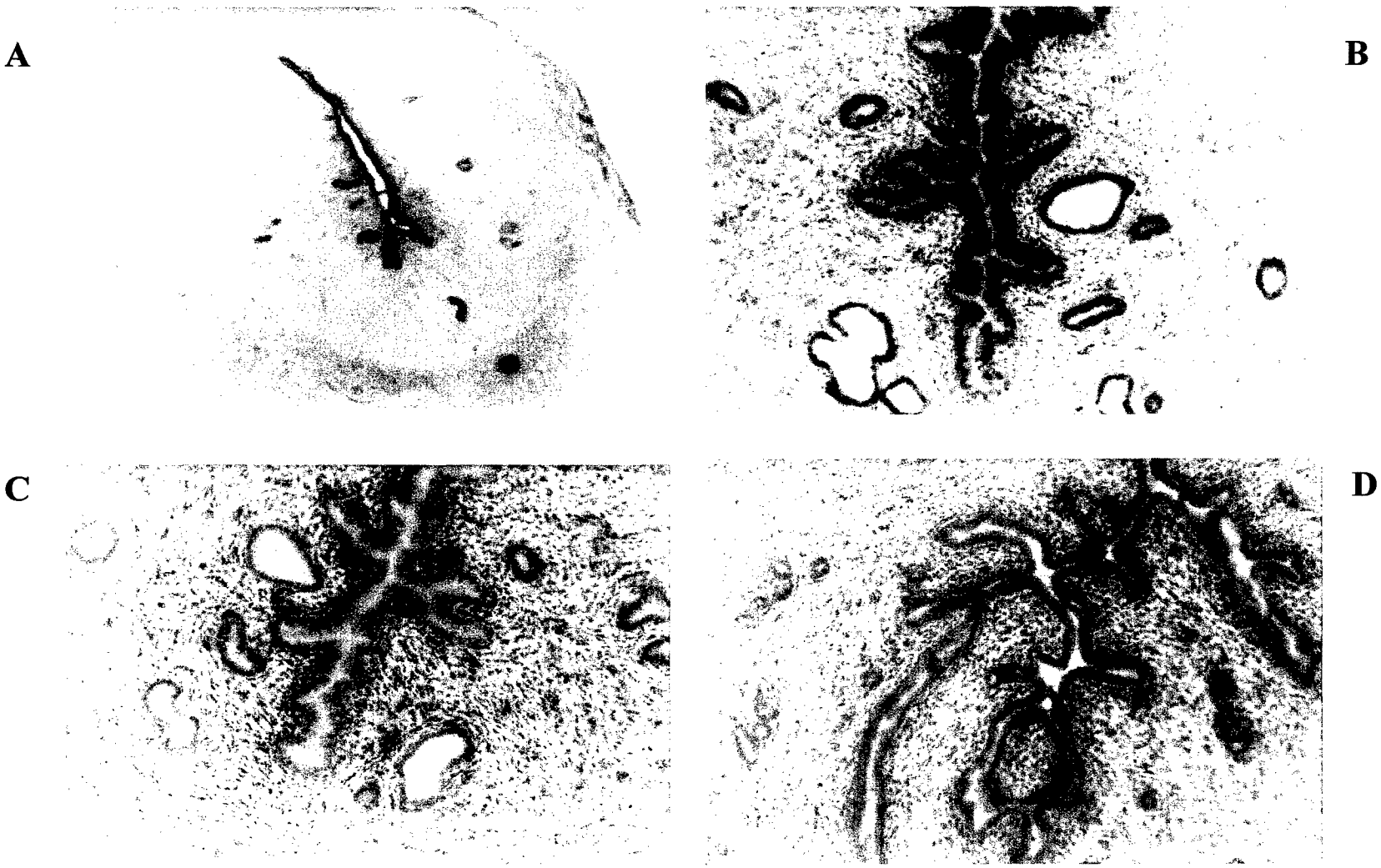

FIGURE 7

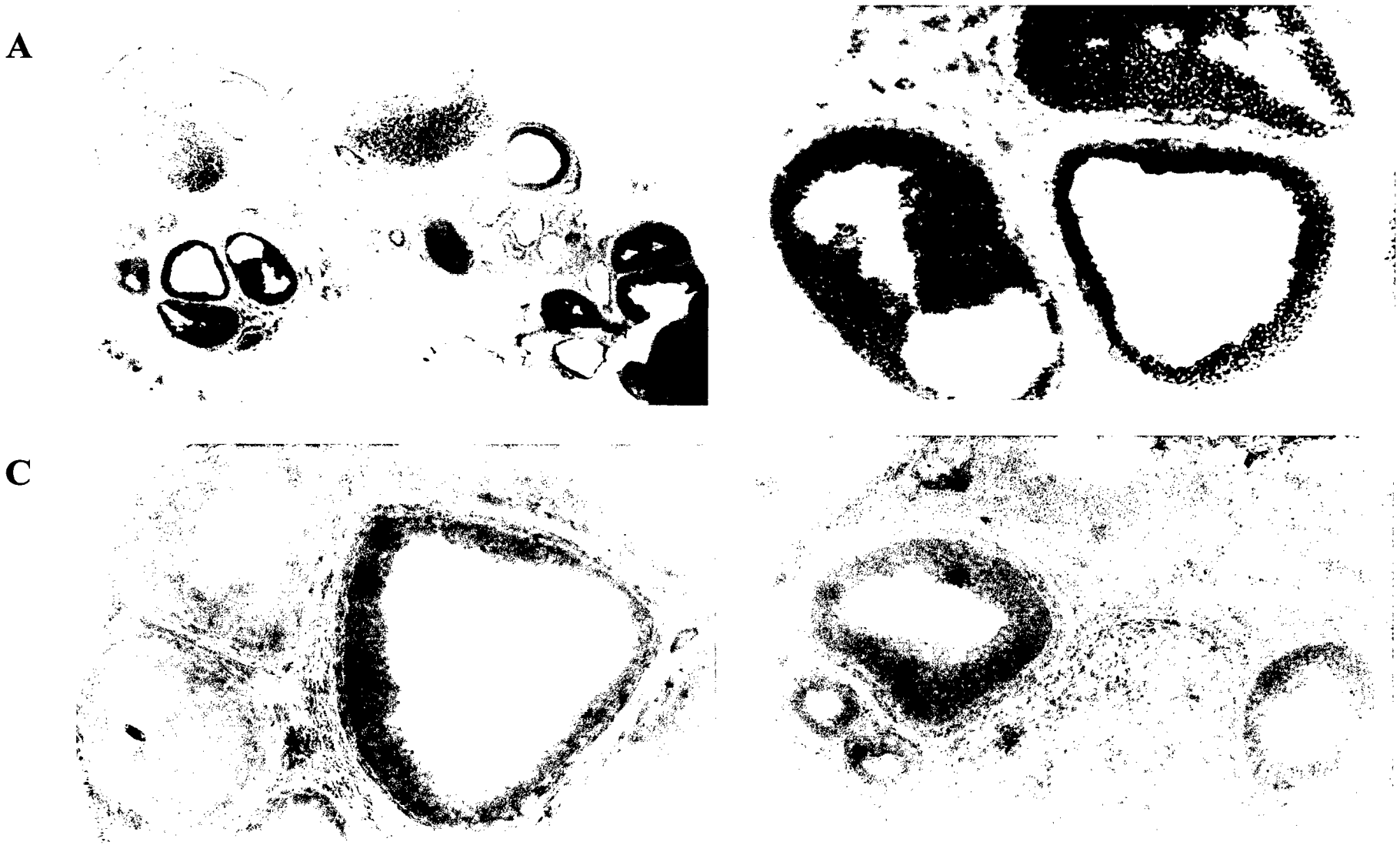

FIGURE 8

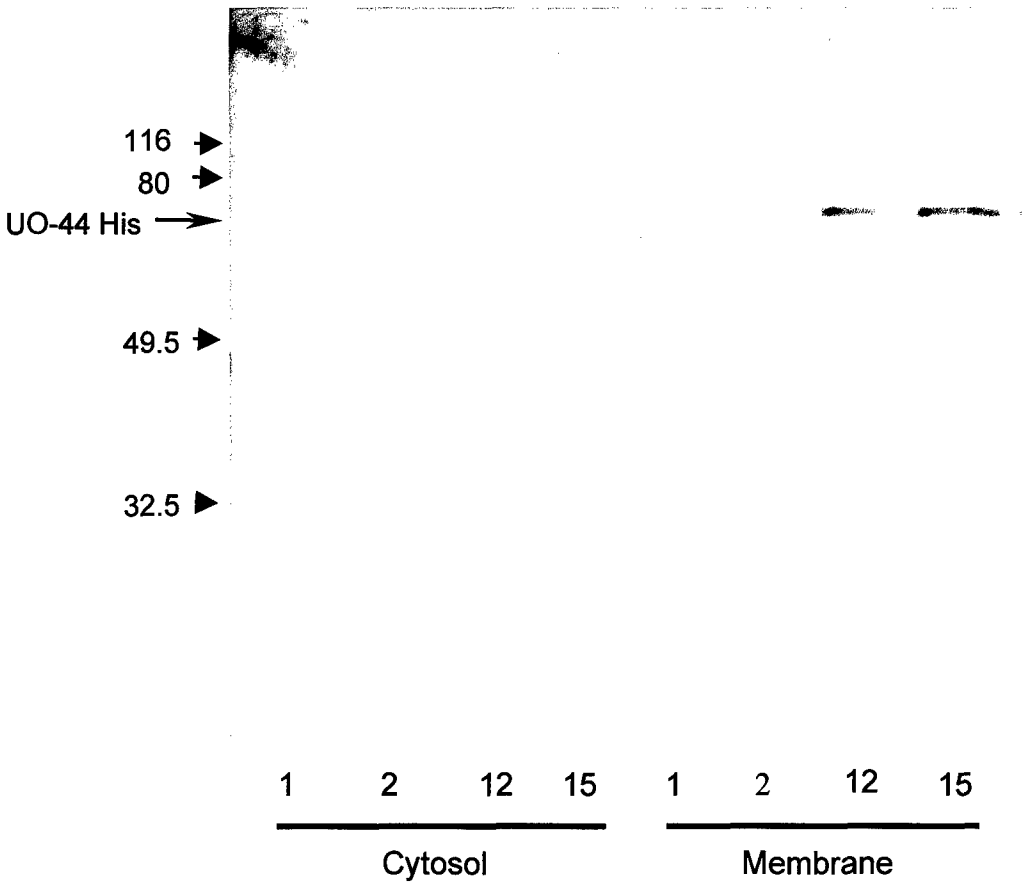

FIGURE 9

Co-administration of finasteride and the pure anti-estrogen ICI 182780 acts synergistically in modulating the IGF-system and PSA in rat prostate

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Running title: Modulation of IGF system by finasteride and anti-estrogen ICI 182780

Abstract

Prostate cancer is the most diagnosed invasive malignancy in males. Androgens and estrogens have been implicated in the pathogenesis of prostate cancer. We report herein that the pure anti-estrogen ICI 182780 reduces Ki-67 labelling index, prostate specific antigen (PSA), androgen receptor (AR), and type I insulin-like growth factor (IGF-I) receptor levels in rat prostate. Increase of IGF-I mRNA and IGF binding protein 3 (IGFBP-3) protein accumulation occur without any effect on prostate weight. Finasteride significantly decreases prostate weight and inhibited IGF-I gene expression. IGFBP-3 mRNA and PSA levels are not affected by finasteride. Co-administration of ICI 182780 plus finasteride reduces prostate weight by approximately 50% and causes acinar dilation with decreased luminal epithelial cell thickness. The acinar epithelial cells became atrophic and inactive with minimal cytoplasm. We also demonstrate a synergistic effect of ICI 182780 and finasteride on reduction of PSA and increase of IGFBP-3 accumulation. Because the IGF and IGFBP-3 system plays an important role in prostate epithelial cell proliferation, apoptosis and tumor progression, the inhibitory effects of finasteride and ICI 182780 on IGF system and PSA may contribute to their anti-proliferative activity. These observations support a potential use of ICI 182780 in conjunction with finasteride in the prevention and/or treatment of prostate cancer.

Introduction

The prostate gland requires androgens for growth, maintenance and function. Androgen-deprivation therapy causes marked and characteristic changes in both normal prostate and prostate cancer (1,2). The 5α-reductase enzyme is responsible for the conversion of testosterone (T) to dihydrotestosterone (DHT) in androgen-dependent target cells (3,4). DHT has a greater affinity for the androgen receptor (AR) than testosterone, and it plays an important role in the regulation of prostatic growth. Finasteride acts as a competitive and specific inhibitor of 5α-reductase, resulting in suppression of serum and intraprostatic DHT concentration to castrated levels, with subsequent reduction in prostatic size (5-7). Finasteride also inhibits AR expression (8). Inhibition of 5α-reductase has been shown to inhibit the growth of prostate cancer both *in vivo* and *in vitro* (9-12). An attractive feature of finasteride is its excellent safety profile (6,7,13-15), making it a reasonable candidate for chemoprevention in high-risk target populations.

Estrogen receptors are present in fibroblasts, basal epithelial and acinar epithelial cells (16). Both estrogens and DHT decrease the rate of cell death (16). Estrogens induce stromal fibroblasts to express EGF-R and FGF-R, increase AR levels and stimulate prostate basal epithelial cells to produce growth factors that act on the epithelial cells (16). In dog, estrogens cause marked stimulation of prostate growth (17). Increased serum estrogens promote prostate growth indirectly by increased growth factor production (16).

Several epidemiological studies provided data suggesting that the incidence of prostate cancer is higher in men with higher circulating IGF-I levels (18,19). IGF-I has been shown to be an anti-apoptotic agent and acts as a mitogen in the prostate gland. Systemic treatment with IGF-I for 7 days resulted in a 29% increase in the net weight of the ventral prostate (20). Persistent

expression of IGF-1 in the basal epithelium of mouse prostate leads to neoplasia in this tissue (21). Suppression of IGF-1 expression by finasteride was correlated with decrease in prostate weight (22). Blocking of IGF-II autocrine loop by vitamin D and its analogue EB1089 inhibits PC-3 prostate cancer cell proliferation (23). The role of IGFs in prostate growth is mediated in part by the interrelated components of the IGF system, which includes IGF receptors, IGFBP receptors and IGFBP proteases. The IGFBPs are a family of seven proteins that bind to IGFs with high affinity (24,25), and control the distribution of IGFs. Normal prostate epithelial cells secrete IGFBP-2 and IGFBP-4, whereas the stromal fibroblasts produce IGFBP-2, 3 and 4 (26,27). Following castration, the expression of IGFBP-2, 3, 4 and 5 genes in the ventral prostate is rapidly induced (28). It has been hypothesised that IGFBPs attenuate the cellular response to IGF-I through the high affinity binding of IGF-I to IGFBPs. This interaction sequesters IGF-I away from its receptor, and hence interfering with the normal homeostatic intracellular signalling downstream of the receptor. It is also known that some IGFBPs have intrinsic bioactivity, which is independent of IGF (29-31). IGFBP-3 can have an inhibitory activity probably through TGF-ß receptor type V (32). Changes in IGFBP-3 levels from benign to malignant disease has been reported (33). In the process of neoplastic progression, activation of autocrine loops is a common event. Neoplastic progression is associated with the expression of IGFBP proteases. In the tumour microenvironment, these would tend to increase IGF bioactivity by cleaving IGFBPs and liberating free IGFs. Interestingly PSA is a proteolytic enzyme that cleaves IGFBP-3 in extracellular fluid (34), and an inverse correlation between PSA and IGFBP-3 levels in bone metastases has been reported (35). Epidemiological observations linking IGF-I to risk of prostate cancer provided potential implications of the IGF system as a target for prevention (18,19). In this study, we report that blockage of estrogen action by a pure anti-estrogen ICI 182780 (36) and reduction of DHT levels by finasteride lead to reduction of IGF-mediated autocrine/paracrine loops and reduction in prostate weight. This approach may offer a clinical utility for prostate cancer and/or benign prostate hyperplasia.

Materials and Methods

Animals. Animal studies were performed in agreement with the guidelines of the local Animal Care. To block the conversion of testosterone to dihydrotestosterone (DHT), male Sprague-Dawley rats (Charles River, Quebec) weighing 300-350 g were treated with 5 mg finasteride/kg body weight/day (5mg/tablet, Merck Frost, Quebec, Canada) by gavage. The finasteride dose was based on our previous studies (22). Control animals received the same volume of water. To block estrogen activity, ICI 182780 (36) (Zeneca Pharmaceuticals) was supplied at a concentration of 50 mg/ml in castor oil solution. Rats received castor oil or either 1 mg or 1.5 mg ICI 182780 per kg body weight per week for 3 consecutive weeks. To investigate the combined effects of ICI-182780 and finasteride, rats were daily treated with 5 mg finasteride/kg body weight plus either 1 mg ICI 182780 or 1.5 mg ICI 182780 per kg body weight per week for 3 consecutive weeks. Animals were sacrificed 21 days following the initial treatment and the prostate tissue was collected and weighed. A portion of the prostate tissue was fixed in 10% buffered formalin for paraffin embedding and the remainder was immediately frozen in liquid nitrogen for extraction of RNA and protein.

Immunohistochemistry and histology: Fixed prostate tissue was routinely processed in a tissue processor and embedded in paraffin. Sections of 5 μm were cut and stained with haematoxylin-eosin. Examination of the slides was performed by light microscopy. The ImmunoCruz Staining System was used for immunohistochemical study. Briefly, the slides were deparaffinized, dehydrated in water and incubated with 3% H_2O_2 for 20 min to block endogenous peroxidase activity. To examine expression of Ki-67, antigens were retrieved by heating the slides in citrate buffer (pH 6) for 5 min. After preincubation with normal serum for 20 min at room temperature, the primary antibody was applied (2 μg/ml) and incubated overnight at 4 ^{0}C. The section was then incubated with the appropriate biotinylated secondary antibody at 1:500 dilution followed by

peroxidase-conjugated streptavidin complex according to the manufacturer's instruction and DAB. The section then counterstained with haematoxylin. Between each change of incubation the sections were rinsed 3 times in PBS for 5 min each. To evaluate the Ki-67 labelling index, 500 epithelial cells were counted for each group in randomly chosen fields at a x 400 magnification. The Ki-67 labelling index was expressed as the number of clearly labelled Ki-67 reactive nuclei in 500 cells counted. Significance difference was determined by Student t-test.

Western Blotting. To determine the changes in the expression of AR, IGF-IR, IGFBP-3, MAPK and PSA proteins, prostate tissue was homogenized in lysis buffer (1 mM $CaCl_2$, 1 mM $MgCl_2$, 1% NP-40, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μM PMSF, and 100 μM $NaVO_4$). Proteins were subjected to Western blot analysis as described (37). Blots were incubated with either rabbit anti-AR (Santa-Cruz), rabbit anti-IGF-IRß (Santa-Cruz), rabbit anti-IGFBP-3 (Upstate Biotechnology, Lake placid, NY), mouse anti-PSA (Santa-Cruz), mouse anti-phospho specific MAPK (New England BioLabs) and mouse anti-α tubulin (Santa-Cruz) antibodies and horseradish peroxidase-conjugated donkey anti-mouse or anti-rabbit secondary antibody (1:7500). Blots were visualized with a chemiluminescent detection system as described by the manufacturer (ECL, Amersham).

Northern Blot: Total RNA was isolated from prostate tissue and Northern blotting was performed as described (38). Blots were hybridized with IGF-I (39), IGF-IR (ATCC), and IGFBP (1-5) (40) cDNAs. To control for equal RNA loading, blots were rehybridized with GAPDH cDNA (ATCC). Quantitative analysis of gene expression was accomplished by scanning autoradiograms and densitometry. For each lane, the sum of the density of bands corresponding to transcripts hybridizing with the probe under study was calculated, and normalized the amount of RNA loaded.

Results

Treatment of rats with a pure anti-estrogen, ICI 182780, for 3 weeks has no effect on prostate weight. Daily treatment of finasteride at a dose of 5 mg/kg body weight caused 25% reduction in prostate weight ($p<0.01$) (Fig. 1). Prostate weight was about 50% of controls when ICI-182780 was co-administered with finasteride ($p<0.01$) (Fig. 1). The maximal dose required for maximal reduction in prostate weight was 1 mg ICI 182780 and 5 mg finasteride per kg body weight.

The effects of ICI 182780, finasteride and the combination on prostate gland morphology are shown in Figure 2. Control prostate gland presents a cluster of epithelial tubules surrounded by connective tissue. The epithelial ducts had a large lumen, lined by tall cubical cells. The luminal cells were large and apparently active (Fig. 2A). There were no apparent differences between vehicle, ICI 182780 and finasteride-treated prostate glands. Co-administration of ICI 182780 and finasteride resulted in marked histological changes of the prostate gland (Fig. 2D). The ICI 182780/finasteride-induced pattern was characterized by an increased size of the lumen. The acini were dilated with thin and atrophic luminal epithelial cells (Fig. 2D).

The effects of treatment on cell morphology were apparent when the sections were viewed at higher magnification. In the control gland, the luminal epithelial cells were tall. Most of nuclei were basally and in some areas the nuclei were arranged in an irregular fashion (Fig. 3A). Both ICI 182780 and finasteride caused a reduction in thickness of epithelial cell (Fig. 3A versus 3B and 3C). Unlike control and finasteride treatments, ICI 182780-treatment resulted in a single layer of tall columnar epithelial cells and nuclei were basal (Fig. 3C). In the finasteride-ICI 182780-treated prostate gland, the epithelial layer lined the lumen was very thin. The epithelial cells were inactive, with diminished cytoplasm (Fig. 3D).

We examined the effect of hormonal regulation of Ki-67 expression in prostate tissue. Figure 4 shows the results of an experiment where prostate tissues were collected from rats treated with vehicle, finasteride, ICI 182780, and ICI 182780 plus finasteride. Both finasteride and ICI 182780 significantly decreased the number of epithelial cells expressing Ki-67 as compared to controls ($p<0.05$). No significant reduction in Ki-67 labelling index of the epithelium was seen when ICI 182780 and finasteride were co-administered as compared with the effect of either ICI 182780 or finasteride alone.

Since over-expression of IGF-I in the basal epithelium of prostate leads to spontaneous hyperplasia (21) and systemic injection of IGF-I increased wet weight of the ventral prostate (20), the *in vivo* effects of ICI 182780, finasteride and finasteride plus ICI 182780 on IGF-I, IGF-I receptor and IGFBP expression were examined. As shown in figure 5, daily treatment of male rats with 5 mg finasteride per kg body weight resulted in 30% decrease in IGF-I mRNA ($p<0.05$). ICI 182780, at a dose of 1.5 mg, caused a 1.8-fold increase in IGF-I transcripts as compared to controls ($p<0.01$). The IGF-I gene expression was significantly inhibited when finasteride and ICI 182780 were co-administered ($p<0.01$). Neither ICI 182780 nor finasteride had significant effects on IGFBP-3 mRNA ($p<0.05$) when administered as a single agent. However, when they were given together, the IGFBP-3 mRNA levels were significantly augmented ($p<0.01$).

The androgen receptor (AR) and IGF-I receptor proteins were significantly decreased by ICI 182780 ($p<0.01$). One mg of ICI-182780 per kg body weight was sufficient to inhibit both AR and IGF-IR expression (Fig. 6). Finasteride had no effect on either AR or IGF-IR levels. When ICI 182780 was co-administered with finasteride, significant inhibition of AR and IGF-IR expression was observed only at a dose of 1.5 mg ICI 182780 per kg BW ($p<0.01$) (Fig. 6). There was a

positive correlation between AR and IGF-IR levels. Despite significant reduction in IGF-IR following ICI-182780 or combined ICI 182780 and finasteride, the levels of MAPK phosphorylation were not significant affected ($P<0.05$) (Fig. 6).

Since PSA is a proteolytic enzyme that cleaves IGFBP-3, the effects of ICI 182780, finasteride and finasteride plus ICI 182780 on PSA and IGFBP-3 levels were examined. Figure 7 shows that PSA levels were significantly low in ICI 182780-treated prostate gland as compared to controls ($p<0.01$). Finasteride exerted no effect on PSA production. Further reduction in PSA production was seen in samples derived from ICI 182780-finasteride combined treatment. Either ICI 182780 or finasteride ($P <0.05$) significantly increased IGFBP-3 level. ICI 182780-induced IGFBP-3 synthesis was dose-dependent. Further increase in IGFBP-3 accumulation was observed in ICI 182780 plus finasteride treated prostate tissues.

Discussion

Both estrogens and androgens are essential for prostate growth. Androgen deprivation therapy is widely used for advanced disease and significant advances have come with LHRH analogues and anti-androgens. We report herein that *in vivo* interruption of estrogen action using a pure anti-estrogen ICI-182780 for 3 weeks had no effect on prostate weight while blockade of the conversion of testosterone to DHT by finasteride reduced prostate weight by approximate 25%. The ability of finasteride to reduce prostate weight was enhanced by combined treatment.

Histological studies revealed that both finasteride and ICI 182780 reduced the thickness of prostate luminal epithelial cells when they were given as a single agent. In contrast to finasteride, the luminal epithelial cells became columnar and a single layer of regular basal nuclei was seen. This pattern is typical of terminally differentiated epithelial cells. The dilated alveolar ducts and prostate epithelial cell atrophy were observed when ICI 182780 and finasteride were given together.

IGF-I receptor, PSA and AR proteins were significantly downregulated by ICI 182780 treatment. When given together with finasteride, the ICI 182780 concentration required to block AR and IGF-IR expression was greater. While reducing IGF-IR levels, ICI 182780 increased IGF-I gene expression and IGFBP-3 protein without affecting IGFBP-3 mRNA. Finasteride at the dose of 5 mg/kg body weight reduced IGF-I gene expression without affecting IGFBP-3 mRNA levels. ICI 182780 plus finasteride inhibited IGF-I gene expression, decreased PSA levels and induced IGFBP-3 mRNA accumulation as well as IGFBP-3 protein. The expression of IGFBP-2, -4 and -5 was not affected by any treatment.

Since the prostate gland requires androgens for growth and function, blocking of androgen action

by reducing AR following ICI-182780 or ICI 182780 plus finasteride treatments would lead to decrease in growth and cellular activity. Furthermore anti-estrogens such as ICI 182780 competes with estrogens for estrogen receptor and evokes a different receptor conformation that results in reduced or no production of estrogen-dependent genes such as EGF, EGF-R and FGF-R (16). Thus, blocking of estrogen activity can also disrupt paracrine production of growth factors that act on the epithelial cells. The lost of autocrine stimulatory activity on the stromal cells or paracrine activity on the epithelium by co-administration of ICI 182780 plus finasteride may inhibit epithelial cell activity as indicated by the decrease in cytoplasmic volume of luminal epithelial cells, with subsequent reduction in the size of the prostate. These observations suggest that the growth promoting effects of estrogens and androgens *in vivo* may be mediated, at least in part, by a local synthesis of growth factors such as IGFs and IGFBP-3, based on the hypothesis that the structure and function of the epithelium of the adult prostatic gland is dependent on epithelial-stromal interactions (41).

Both prostate cancer and benign prostate stromal cells have IGF-I receptors, and stromal cells produce IGF-I in response to androgen (42). IGF-I could act via an autocrine stimulatory activity on the stroma cells, or paracrine activity on the epithelium. Inhibition of AR expression leads to reduction in IGF-I production, which in turn reduces epithelial cell proliferation. Since IGF-IR has been shown to play an important role in metastasis of prostate cancer cells (43) and tumorigenesis (21), inhibition of IGF-IR by ICI 182780 and ICI 182780 plus finasteride observed in this study is important because such treatments may suppress tumour growth and reduce or abolish tumour invasion (43). Inhibition of IGF-IR also disrupts IGF-II autocrine loop, which is quite common in prostate cancer. It remains to determine whether a decrease in IGF-IR and IGF-I expression does occur in prostate tumours following ICI 182780/finasteride treatment.

At the present time, it is unknown whether ICI 182780 acts on prostate cells through estrogen receptor α or ß. Our data suggest that in the prostate AR and IGF-IR expression are estrogen-dependent while IGF-I expression is androgen-dependent. Blocking of ER activity leads to reduction in prostate epithelial cell activity as determined by reduction in Ki-67 and PSA. Induction of IGF-I gene expression following ICI 182780 treatment may provide in part an explanation for the failure of ICI 182780 to decrease prostate weight. Increase in levels of testosterone and decrease dihydrotestosterone following finasteride treatment has been reported (22). Thus, a slight decrease in prostate weight following finasteride administration may be due to conversion of testosterone to estrogens, which in turn maintain AR and androgen activity.

The observation that ICI 182780 when co-administered with finasteride downregulates PSA expression but upregulates IGFBP-3 accumulation is significant given the mitogenic activity of IGF-I, the proteolytic activity of PSA toward IGFBP-3, and anti-proliferative activity of IGFPB-3. The changes in IGF-I and IGFBP-3 following anti-estrogen-androgen-based therapies was reported here to be similar to castration (28) and vitamin D analogues-induced apoptosis in rat prostate gland (44). Reduction of PSA, which is also regulated by androgens, resulted in more IGFBP-3, which prevents the interaction of IGF-I with its receptor and decrease mitogenic activity. Furthermore IGFBP-3 may inhibit prostate epithelial cells through TGF-ß receptor type V (32). In normal prostate gland the above changes may be biologically insignificant because PSA is confined to the lumina of epithelial acini and epithelial cells are terminally differentiated and incapable to proliferate. In malignant tissue, in contrast, the acinar arrangement of normal gland is lost, and PSA may leak into the surrounding stroma. If PSA cleaves IGFBP-3 then the IGF-independent growth inhibition imposed by IGFBP-3 on prostate cancer cells is lost. Furthermore, the release of free IGF following IGFBP-3 proteolysis at the tumour site could have direct mitogenic activity on the prostate cancer cells in this area.

Combined treatments have been shown to improve survival (45) and disease-free survival (46) in prostate cancer patients as compared to monotherapy. Several other studies have confirmed the benefit of adding early adrenal androgen blockage to medical or surgical castration (47-49). Other studies have failed to show survival advantages at early analysis (50,51). Our present study shows that ICI 182780 acts synergistically with finasteride to reduce prostate weight, IGF bioavailability and PSA production. These observations may provide inside the fundamental mechanisms by which estrogens influence normal prostate growth, androgen-independent growth and metastasis of prostate cancer cells. They provide a novel combined therapy for BPH and prostate cancer.

Acknowledgements

We would like to thank Drs. A. Wakeling and B. Vose (Astra-Zeneca Pharmaceuticals) for pre-formulated ICI 182780, Dr. Jacques Courville (Merck Frosst) for Proscar, Dr. R.J. Epstein critical reading and helpful discussion of the manuscript. This work was supported in part by grants from the USAMRMC grant number DAMD17-97-1-7084 and Medical Research Council of Singapore to Hung Huynh.

Figure Legends

Figure 1. Effects of ICI 182780, finasteride and ICI 182780 plus finasteride on rat prostate weight. Rats were treated with the indicated concentrations of finasteride (n=10), ICI 182780 (n=10) and ICI 182780 plus finasteride (n=12) for 3 weeks. Prostate tissues were collected, weighed individually and plotted. Bars with different letters are significantly different from one another at $P<0.01$). Data are expressed as the mean + SEM.

Figure 2. Prostate gland morphology in rats treated with vehicle (A), ICI 182780 1.5 mg/kg BW/week (B), finasteride (daily 5 mg/kg BW) (C), or finasteride plus ICI 182780 (D). Note dilation of prostate gland. The prostate epithelial layer was very thin in ICI-182780 plus finasteride treatment. Haematoxylin-eosin stain was used; magnification, x 800.

Figure 3. Prostate gland histology in rats treated with vehicles (A), ICI 182780 (1.5 mg/kg/week) (B), Finasteride (5 mg/kg BW /day) (C) or ICI 182780 (1.5 mg/kg/week) plus Finasteride (5 mg/kg BW /day). Note the basal nuclei seen in ICI-182780-Treated gland (**B**). Finasteride plus ICI-182780 caused acinar dilation. Luminal epithelial cells were atrophic and lost of secretory activity. Haematoxylin-eosin stain was used; magnification. x 1600.

Figure 4. Effects of treatment with ICI 182780, finasteride or ICI-182780 plus finasteride on proliferation of prostate epithelial cells. Rats were treated with indicated dose of ICI 182780, finasteride and finasteride plus ICI-182780 as described under Materials and Methods. Ki-67 labelling index of epithelial cells was determined as described under Materials and Methods. Bars with different letter are significantly different from one another at $P<0.01$). Data are expressed as the mean + SEM.

Figure 5. Effects of ICI 182780, finasteride and finasteride plus ICI-182780 on the expression of IGF-I and IGFBPs in the prostate gland. Rats were treated with indicated dose of ICI 182780, finasteride and finasteride plus ICI-182780 as described under Materials and Methods. Total RNA derived from prostate gland was subjected to Northern blot analysis. Blots were hybridized with IGF-I, IGFBPs (2-5) and GAPDH cDNAs (**A**). Densitomitric scanning of the IGF-I (n=8) and IGFBP-3 (n=8) bands is shown in (**B**). Representative samples are shown.

Figure 6. Effects of ICI 182780, finasteride and ICI 182780 plus finasteride on androgen receptor, IGF-I receptor and MAPK phosphorylation levels. Rats were treated with indicated dose of ICI 182780, finasteride and finasteride plus ICI 182780 as described under Materials and Methods. Proteins extracted from prostate glands were analyzed by Western blot analysis as described under Materials and Methods. Blots were incubated with rabbit anti-androgen receptor (**AR**), rabbit anti-IGF-I receptor ß (**IGF-IRß**), mouse anti-phospho specific MAPK (**Phospho MAPK p44/42**) and anti-mouse α-tubulin (**α-Tubulin**) antibodies and horseradish peroxidase conjugated secondary antibodies (**A**). Blots were visualized with a chemiluminescent detection system. Densitomitric scanning of the AR (n=8), IGF-IRß (n=8) and phospho MAPK (n=8) bands is shown in (**B**). Representative samples are shown.

Figure 7. Effects of ICI 182780, finasteride and ICI 182780 plus finasteride on prostate specific antigen and IGF binding protein 3. Rats were treated with indicated dose of ICI 182780, finasteride and finasteride plus ICI 182780 as described under Materials and Methods. Proteins extracted from prostate glands were analyzed by Western blot analysis as described under Materials and Methods. Blots were incubated with mouse anti-prostate specific antigen (**PSA**), rabbit anti-IGFBP-3 (**IGFBP-3**), and anti-mouse α-tubulin (**α-Tubulin**) antibodies and horseradish

peroxidase conjugated secondary antibodies (**A**). Blots were visualised with a chemiluminescent detection system. Densitomitric scanning of the PSA (n=8) and IGFBP-3 (n=8) bands is shown in (**B**). Representative samples are shown.

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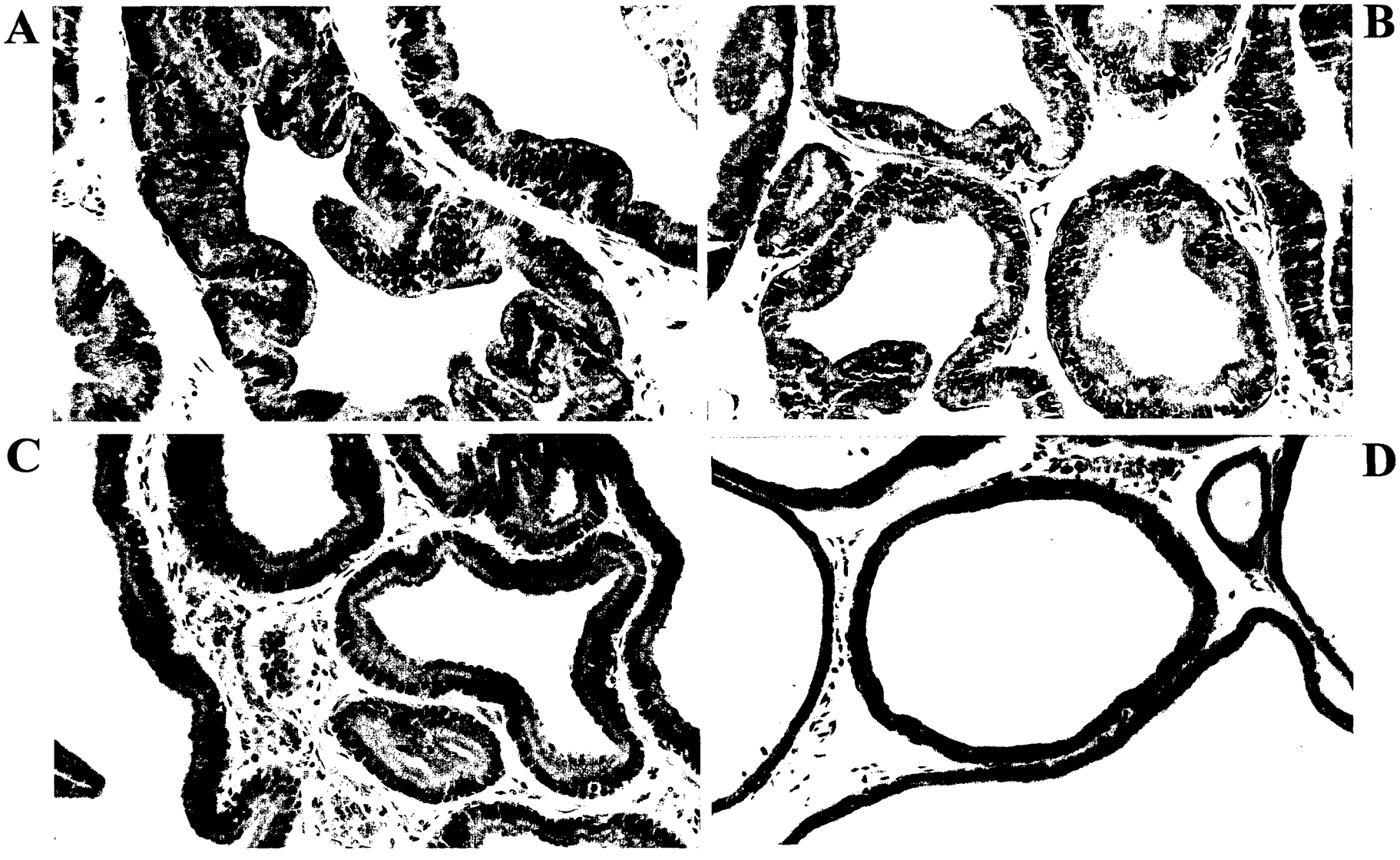


Figure 2

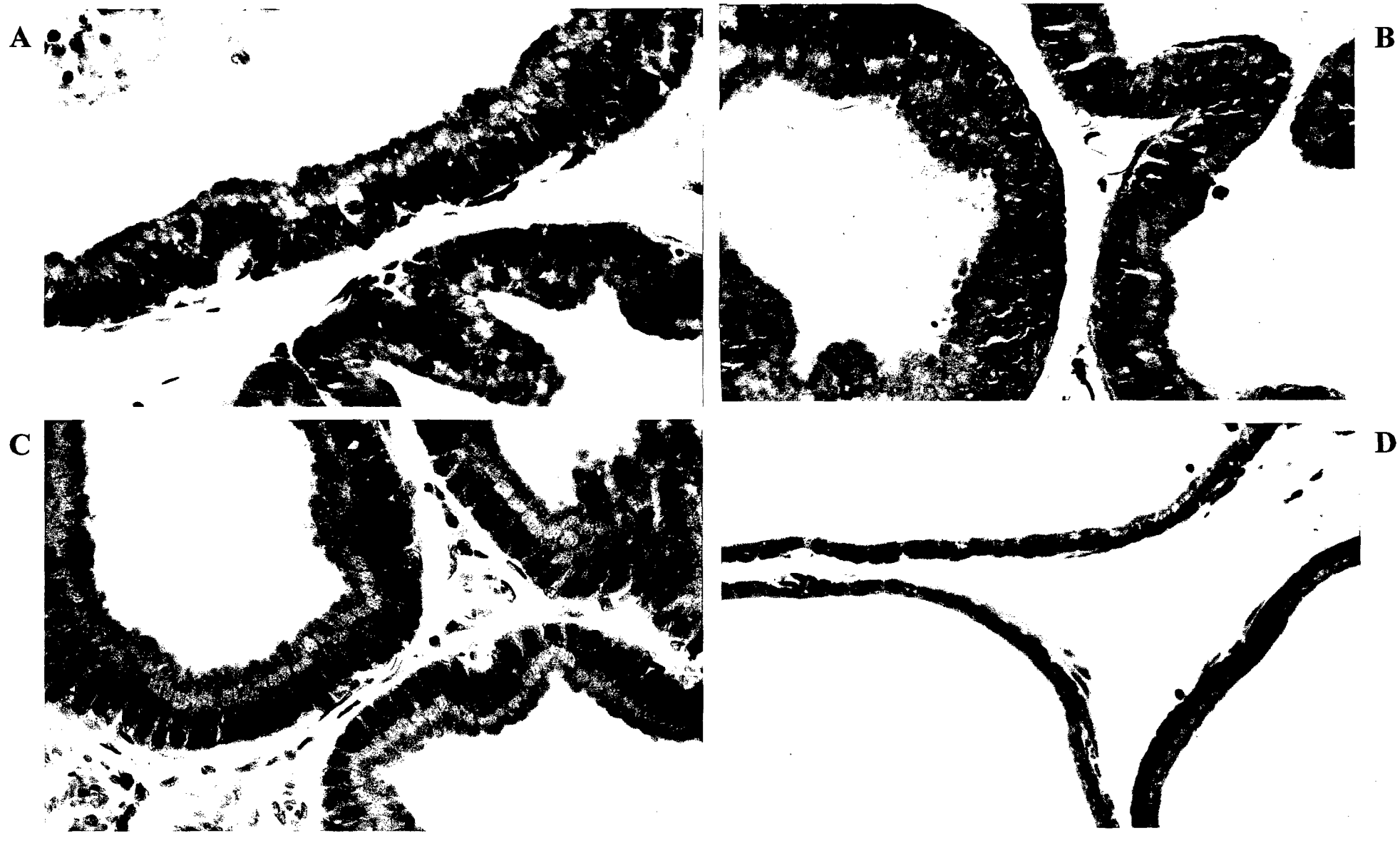


Figure 3

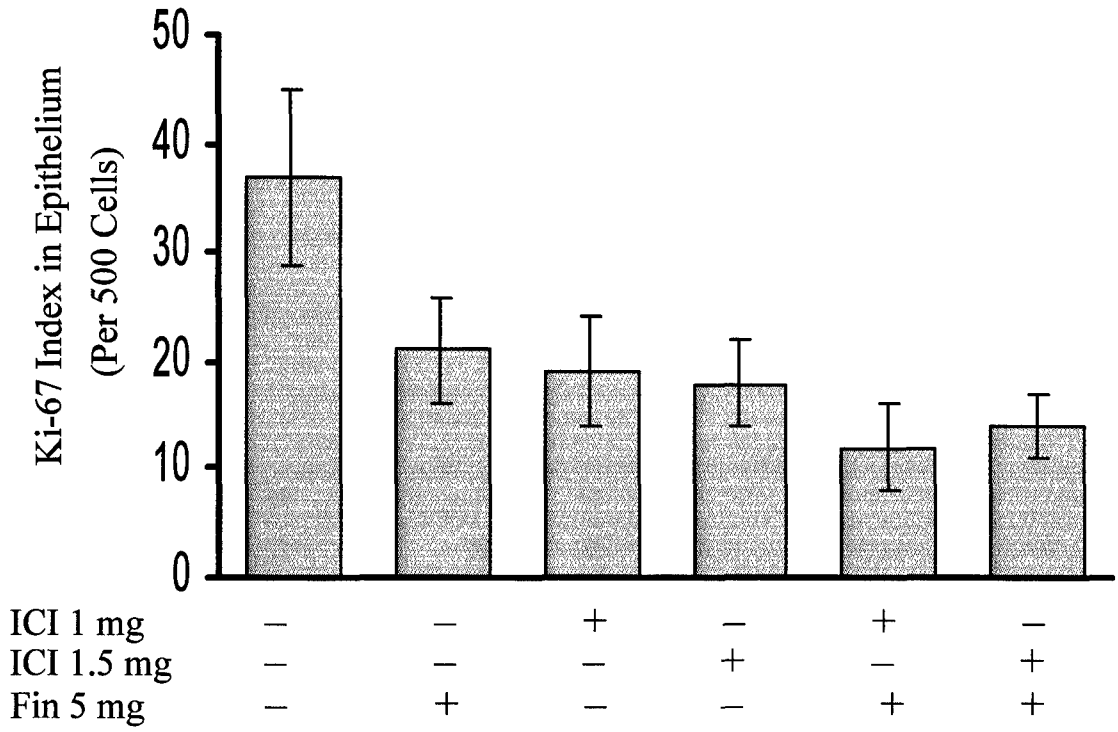
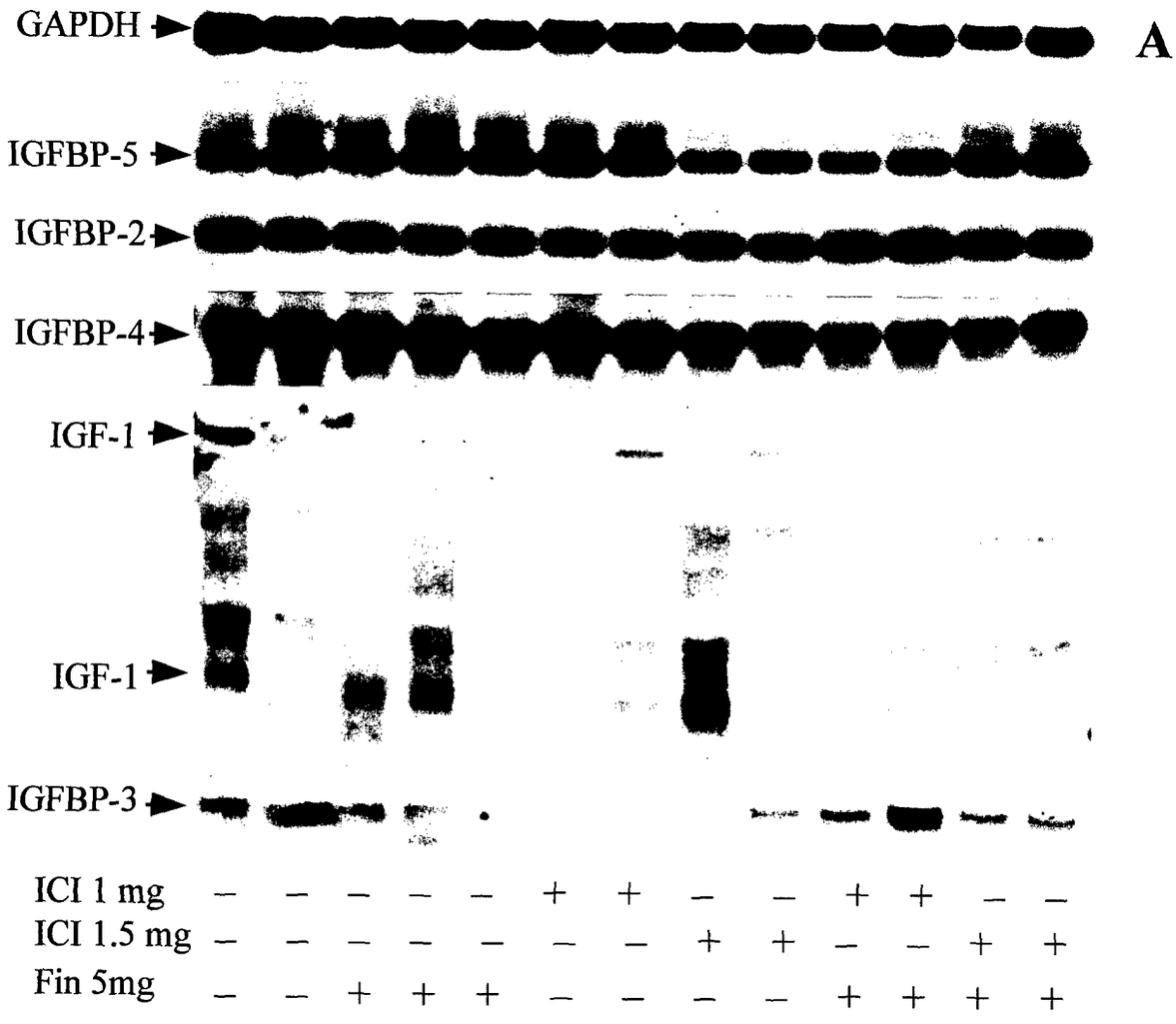

Ki-67 Index in Epithelium
(Per 500 Cells)
50
40
30
20
10
0
ICI 1 mg – – + – + –
ICI 1.5 mg – – – + – +
Fin 5 mg – + – – + +

FIGURE 4


GAPDH
A
IGFBP-5
IGFBP-2
IGFBP-4
IGF-1
IGF-1
IGFBP-3
ICI 1 mg – – – – – + + – – + + – –
ICI 1.5 mg – – – – – – – + + – – + +
Fin 5mg – – + + + – – – – + + + +

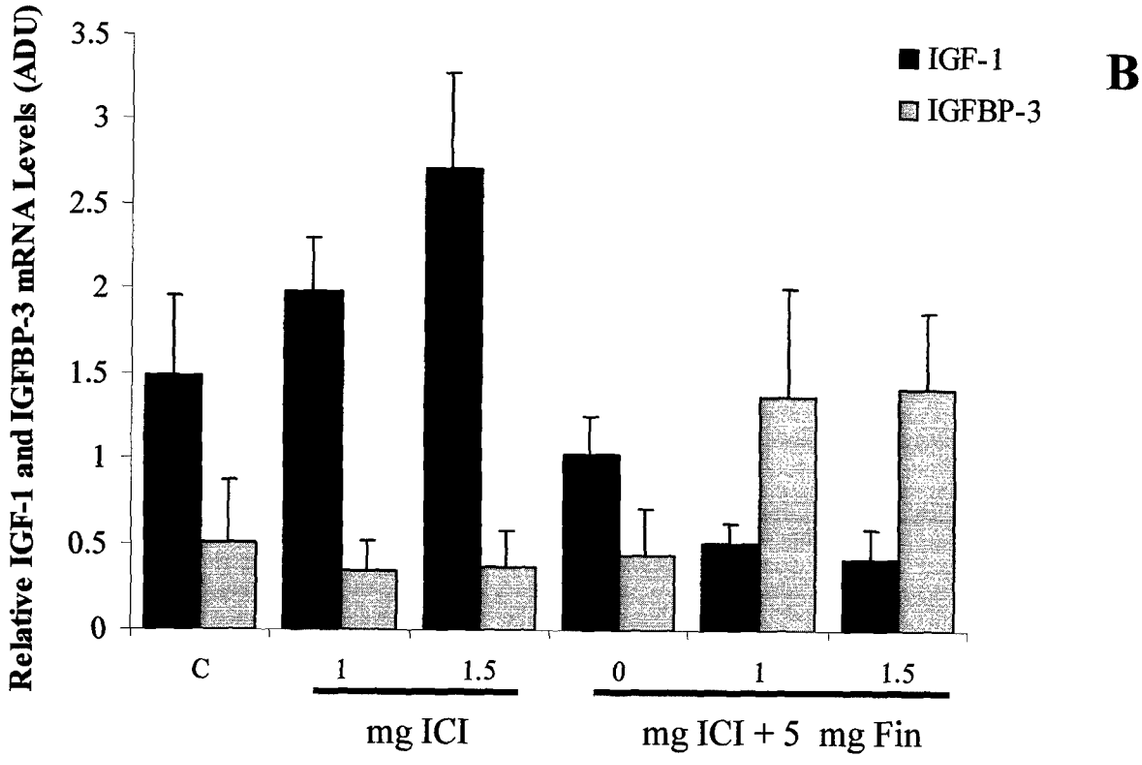
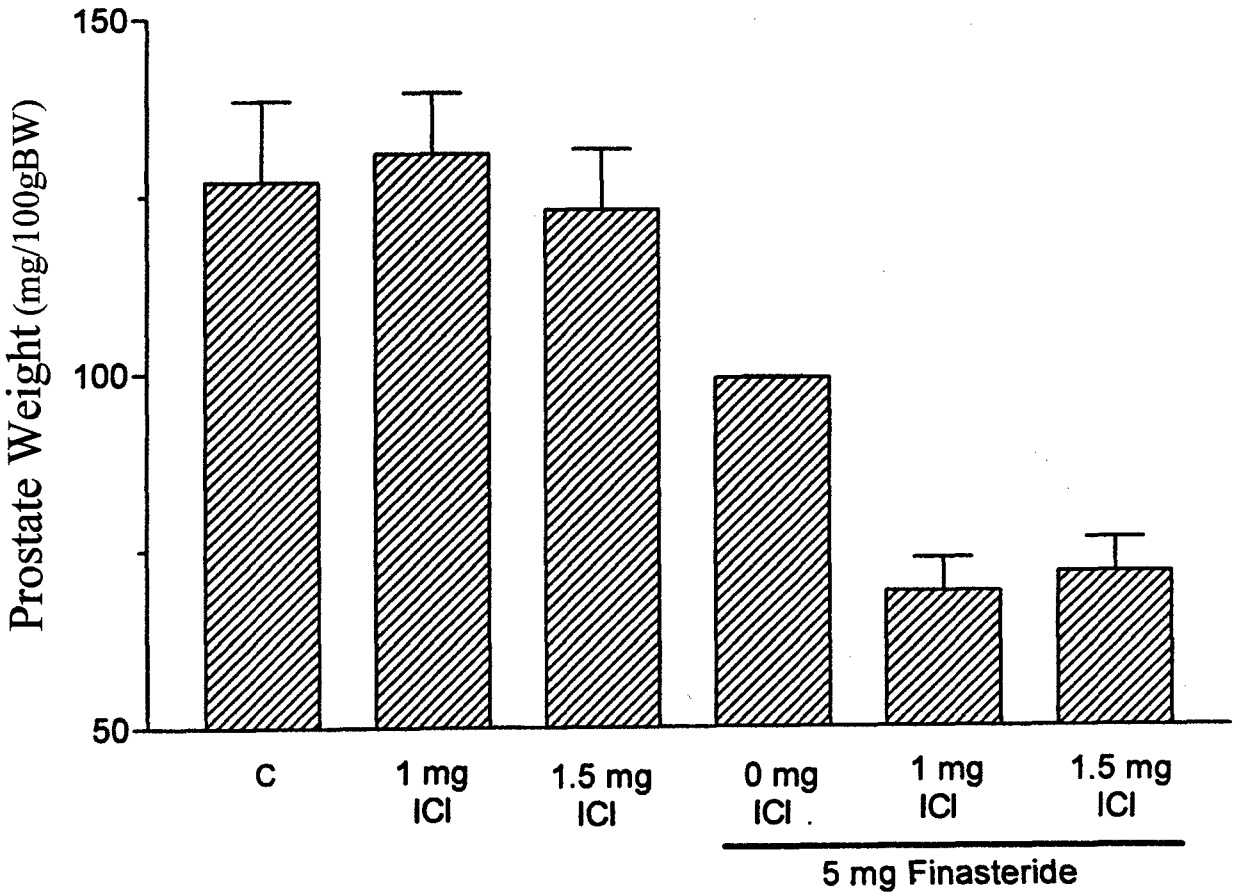
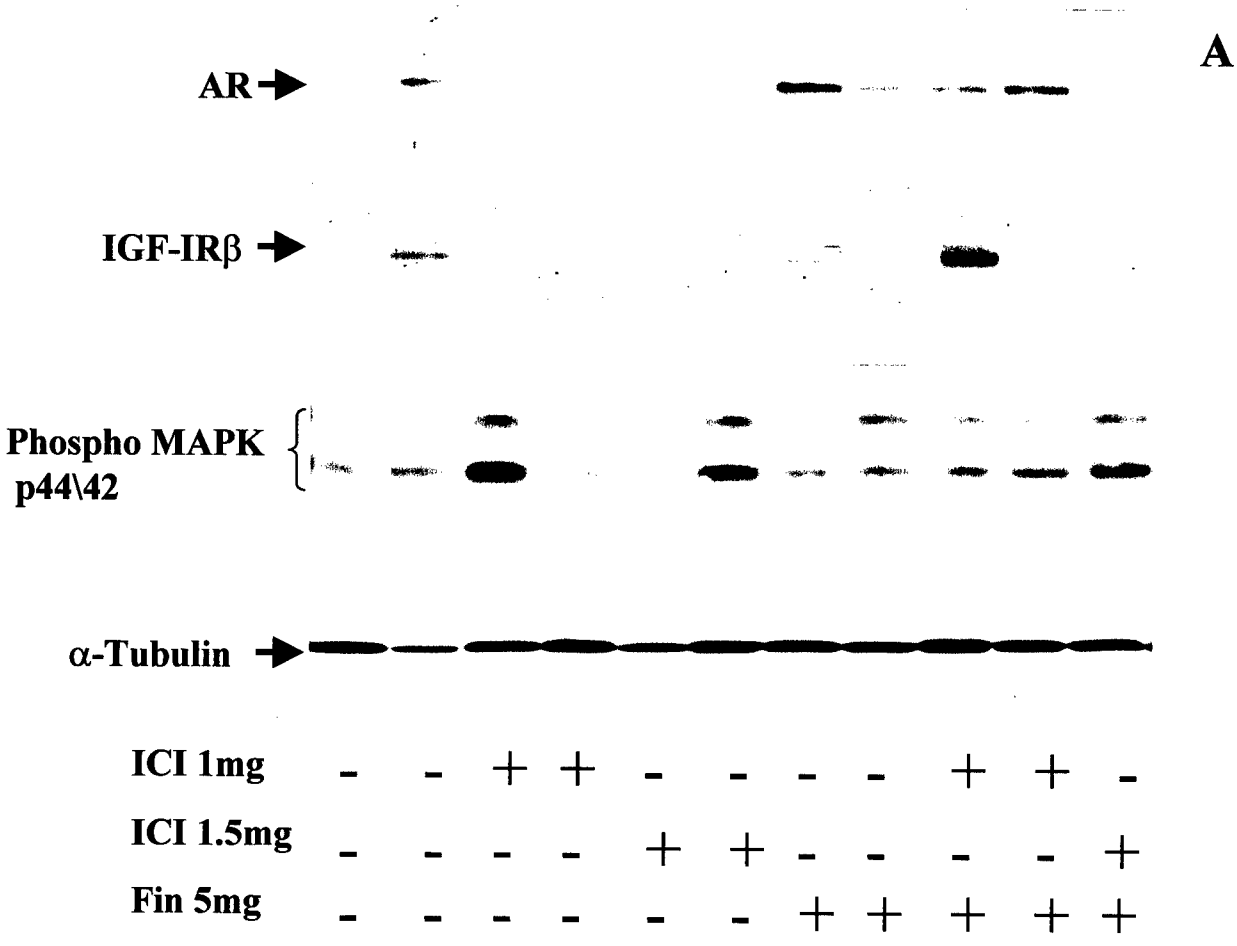

B
IGF-1
IGFBP-3
Relative IGF-1 and IGFBP-3 mRNA Levels (ADU)
3.5
3
2.5
2
1.5
1
0.5
0
C
1
1.5
0
1
1.5
mg ICI
mg ICI + 5 mg Fin

FIGURE 5

FIGURE 1


150
100
50
Prostate Weight (mg/100gBW)
C
1 mg ICI
1.5 mg ICI
0 mg ICI
1 mg ICI
1.5 mg ICI
5 mg Finasteride


A
AR
IGF-IRβ
Phospho MAPK
p44\42
α-Tubulin
ICI 1mg - - + + - - - - + + -
ICI 1.5mg - - - - + + - - - - +
Fin 5mg - - - - - - + + + + +

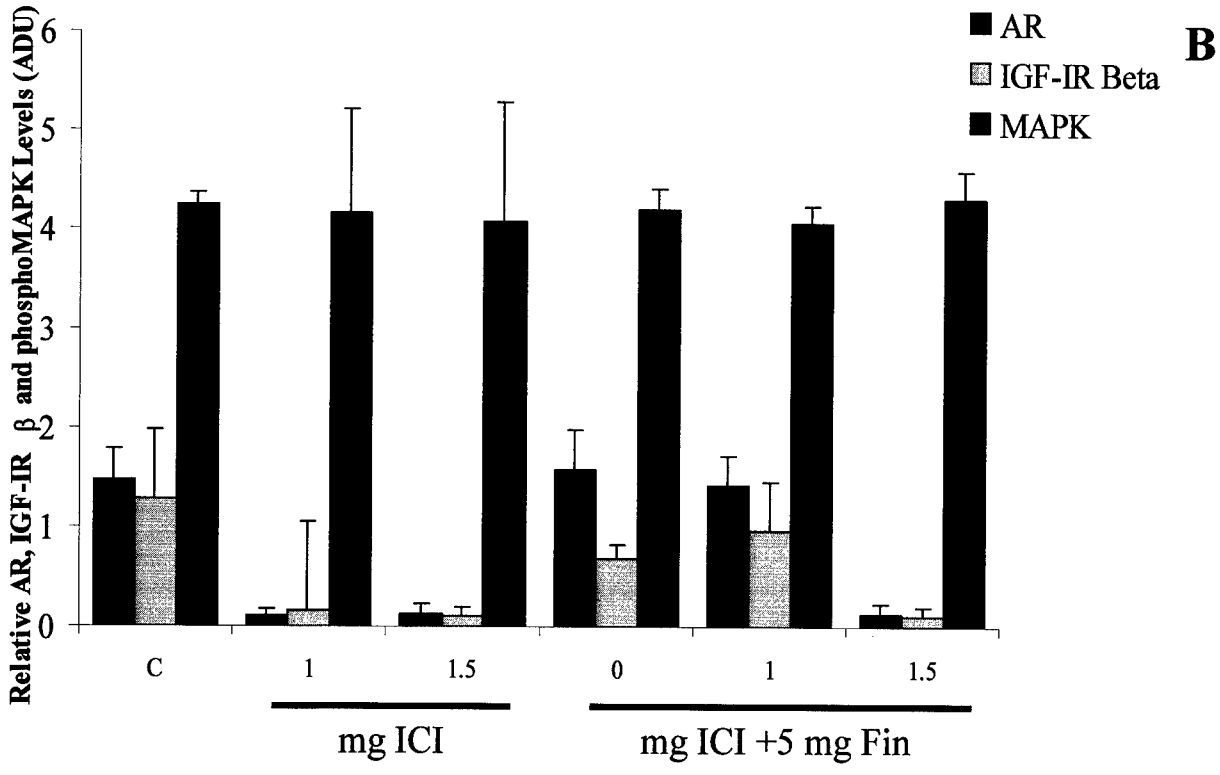
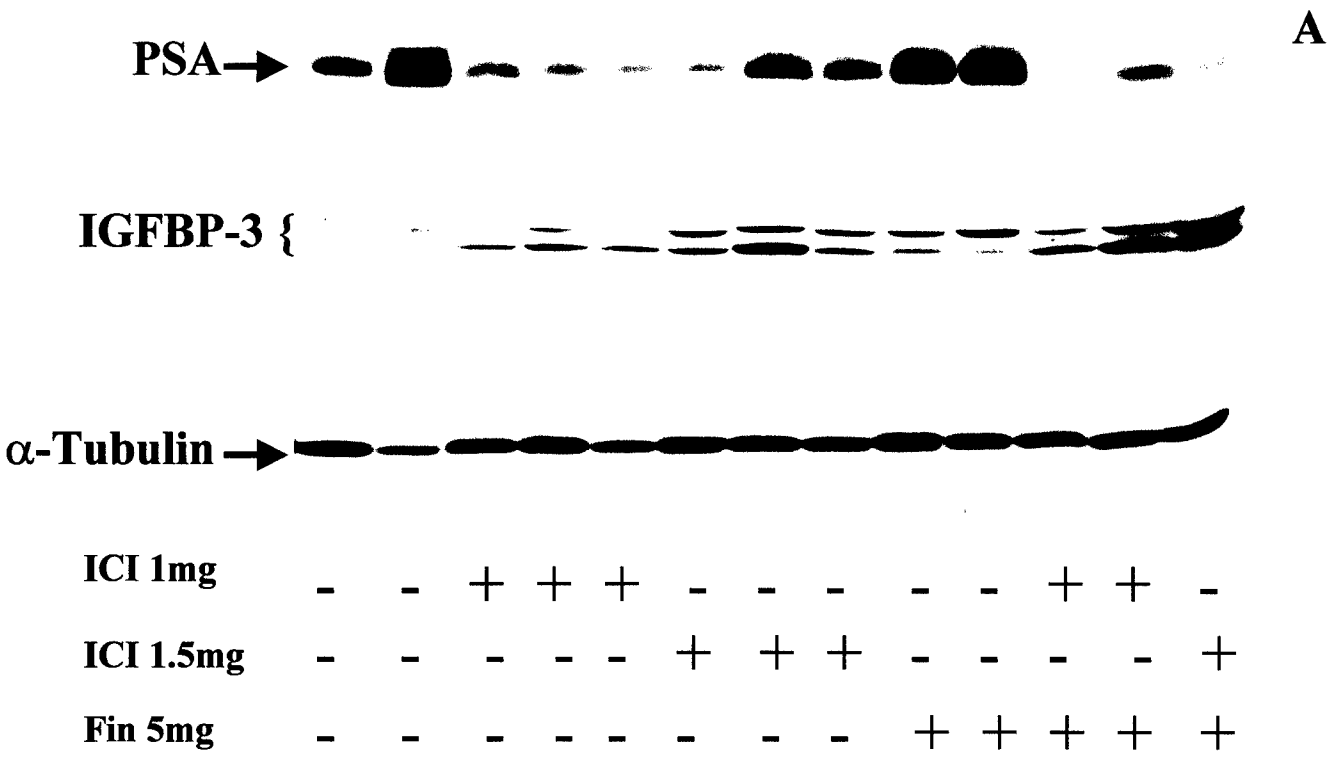

B
AR
IGF-IR Beta
MAPK
Relative AR, IGF-IR β and phosphoMAPK Levels (ADU)
0
1
2
3
4
5
6
C
1
1.5
0
1
1.5
mg ICI
mg ICI +5 mg Fin

FIGURE 6


A
PSA
IGFBP-3 {
α-Tubulin
ICI 1mg - - + + + - - - - - + + -
ICI 1.5mg - - - - - + + + - - - - +
Fin 5mg - - - - - - - - + + + + +

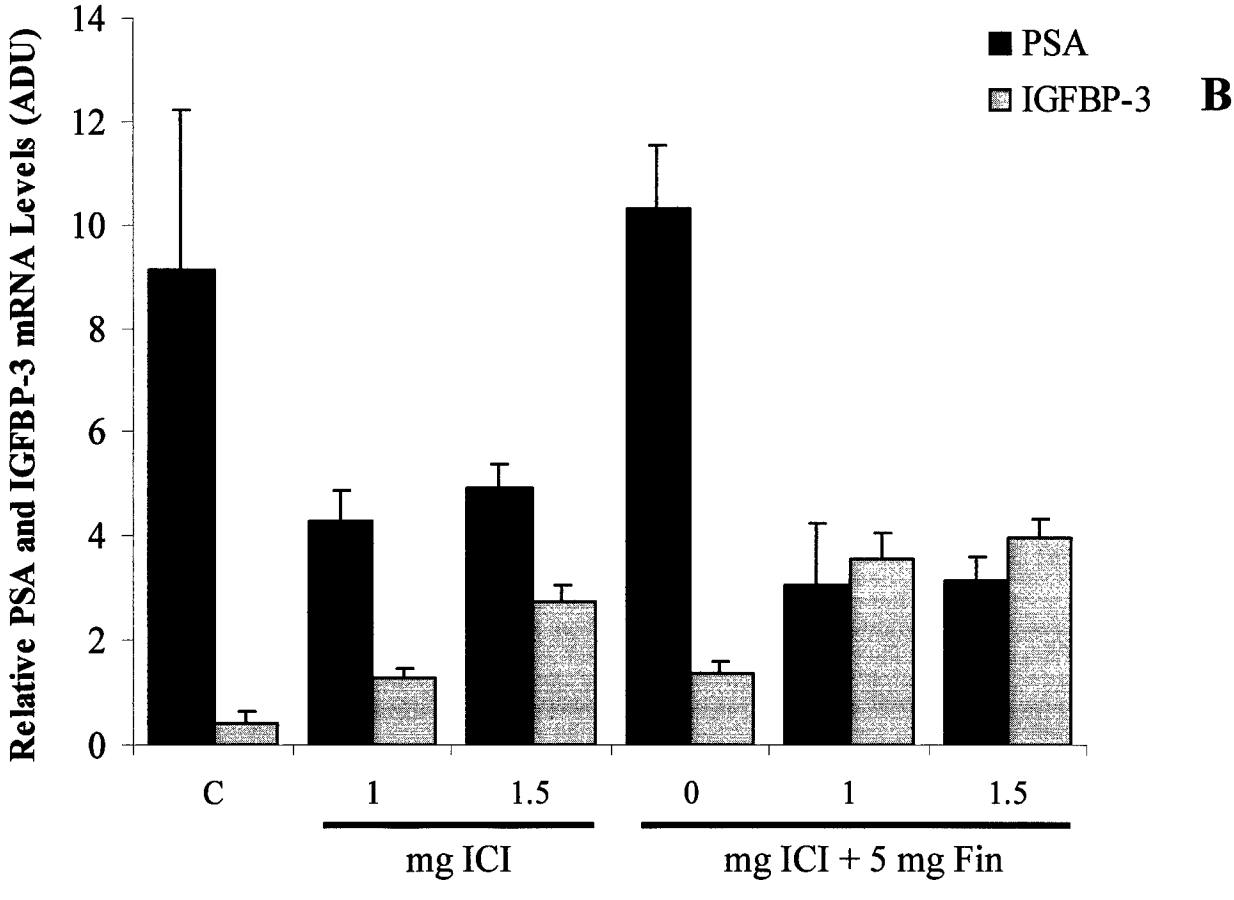

B
PSA
IGFBP-3
Relative PSA and IGFBP-3 mRNA Levels (ADU)
0
2
4
6
8
10
12
14
C
1
1.5
0
1
1.5
mg ICI
mg ICI + 5 mg Fin

FIGURE 7

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EDUCATION

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1990-1994 Ph.D., McGill University (Molecular Biology)
1988-1990 M.Sc., McGill University (Molecular and Cell Biology)
1985-1988 B.Sc., McGill University (Cell Biology)
1983-1985 D.C.S., CEGEP Vanier College, Montreal, Canada (Health Sciences)

ACADEMIC POSITIONS

1999 -curr. Principal Investigator of Laboratory of Molecular Endocrinology; Project Director of Program in Drug-directed Hormonal Therapy of Breast and Prostate Cancers, National Cancer Centre, Singapore.

1999-curr. Adjoint Professor in Department of Medicine and Department of Surgery, Project Director, Lady Davis institute for Medical Research, McGill University

1996-1998 Assistant Professor in Department of Urology, Department of Medicine , Project Director, Lady Davis institute for Medical Research, McGill University

1994-1995 Assistant Professor, Project Director, Lady Davis Institute for Medical Research, McGill University

PROFESSIONAL MEMBER

American Association for Cancer Research
The Endocrine Society

AWARDS

1997-2000 **The United States Army Medical Research and Materiel Command Breast Cancer Research Program Award**
1997-2000 **Fonds de la Recherche en Sante du Quebec Chercheur-boursier Award**
1998 **The 4th Asian Congress in Urology Best Paper Award**
1998 **International Biology of Prostate Growth Symposium Award**
1995 **Canadian Breast Cancer Foundation Award**
1994 **Dean's Honour List (Ph.D. Degree)**
1991-1992 **Natural Scientific and Engineering Research Council Award (NSERC)**
1990-1991 **NSERC**
1989-1990 **NSERC**
1988-1989 **NSERC**

1987-1988 **McConnell**
1986-1987 **McConnell**
1986-1987 **Eliza M. Jones**
1985-1986 **Eliza M. Jones**

PUBLICATIONS

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1. **Huynh**, H., Chan, T, W., Ng, C. Y. and Fouti, N. (2000) Characterization of a novel tamoxifen-induced cDNA (UO-44) in the rat uterus and ovary. (Submitted).

2. **Huynh**, H. (2000). Testosterone enanthate induced breast cancer cell proliferation is associated with phosphorylation of Rb p110 and activation of MAP kinase. 2000. (Submitted).

3. **Huynh**, H. (2000). Pure anti-estrogen and 5α-reductase inhibitor combination in prevention and /or treatment of prostate cancer. (Submitted).

4. **Huynh**, H. (2000). Inhibition of androgen receptor expression in the prostate gland by pure anti-estrogen ICI 182780. (Submitted).

5. **Huynh**, H. (2000). Inhibition of Insulin-like Growth Factor Signaling Pathways in Mammary Gland by Pure Anti-estrogen ICI 182780. (Submitted).

6. **Huynh**, H. (2000). Antiestrogen-induced apoptosis and alteration of the insulin-like growth factor system of mitogens in mammary gland. (Submitted).

7. **Huynh**, H. (2000). Cloning and characterization of a novel pregnancy-induced growth inhibitor in the mammary gland. (Submitted)

8. Marcantonio, D., Moulay, A., Chalifour, L., and **Huynh**, H. (2000). Cloning and characterization of a novel gene that is tightly regulated by estrogen and is associated with mammary gland tumours. (submitted).

9. **Huynh**, H. (2000). Absence of estrogen activity is permissive conditions for androgen to induce mammary epithelial cell differentiation. (submitted)

PATENTS

1. **Immortalized Bovine Mammary Epithelial Cell Lines** (American Type Culture Collection # CRL 10274) United States Patent 5,227,301, issued July 13, 1993. Joint inventor: Dr. J.D. Turner.

   This patent covers the establishment of a series of bovine mammary epithelial cells (denoted MAC-T, Mammary Alveolar Cells-SV 40 large T antigen) that are useful in in vitro diagnostic systems for screening eukaryotic gene expression of DNA constructs. These cells are also useful in the production of authentic proteins using eukaryotic fermentation. (NSERC guideline 235; funded by McGill University).

2. **Gene for Mammary Derived Growth Inhibitor (MDGI)** (Submitted July 31, 1995 to United States Patent Office). Joint inventor: Dr. M. Pollak.

   The MDGI gene has been shown to have tumour suppressor properties in neoplastic mammary cell lines. The MDGI gene and the protein gene product of the gene may be of potential use in detection, diagnosis, evaluation or therapy of breast cancer. There is also a potential for use in evaluating the risk of developing breast cancer.

Patents applied

1. Chalifour, E.L. and **Huynh**, T.H. Steroid-hormone responsive gene expressed in heart and therapeutical uses. (Patent pending)

2. **Huynh**, H. Pure anti-estrogen ICI 182780 and 5α-reductase inhibitor combination in prevention and /or treatment of prostate cancer (Patent applied).

3. **Huynh**, H. Terminal differentiation of breast epithelial cells by pure anti-estrogen ICI 182780 and testosterone enanthate: A novel approach in breast cancer prevention and/or treatment. (Patent applied).

4. **Huynh**, H. and Marcantonio, D. Steroid sensitive gene 1 (SSG1) is a novel estrogen-induced tumour marker. (Patent applied).

ABSTRACTS

1. **Huynh**, H. Cloning and characterization of a novel pregnancy-induced growth inhibitor in mammary gland. DoD Breast Cancer Research Program: Era of Hope Meeting. Atlanta, Georgia. June 8-12, 2000.

2. **Huynh**, H. Cloning and chracterization of a novel pregnancy-induced growth inhibitor in the mammary gland. AACR, San Francisco, CA. 2000.

3. **Huynh**, H. Inhibition of insulin-like growth factor signaling pathways in mammary gland by pure anti-estrogen ICI 182780. AACR, San Francisco, CA. 2000.

4. Marcantonio, D., Moulay, A., Chalifour, L., and **Huynh**, H. Cloning and characterization of a novel gene that is tightly regulated by estrogen and is associated with mammary gland tumours. AACR, San Francisco, CA. 2000.

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DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

1 JUN 2001

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports. Request the limited distribution statement for reports on the enclosed list be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management

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